RAD5a and *REV3* Function in Two Alternative Pathways of DNA Damage Tolerance in Arabidopsis

A Thesis Submitted to the College of
Graduate Studies and Research
In Partial Fulfillment of the Requirements
For the Degree of Master of Science
In the Department of Microbiology and Immunology
University of Saskatchewan
Saskatoon

By

Sheng Wang

PERMISSION TO USE

In presenting this thesis in partial fulfilment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis. Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Department of Microbiology and Immunology

107 Wiggins Road

University of Saskatchewan

Saskatoon, Saskatchewan, S7N 5E5

ABSTRACT

DNA-damage tolerance (DDT) in yeast is composed of two parallel pathways and mediated by sequential ubiquitination of proliferating cell nuclear antigen (PCNA). While monoubiquitination of PCNA promotes translesion synthesis (TLS), which is dependent on low fidelity polymerase ζ (Pol ζ) composed of a catalytic subunit Rev3 and a regulatory subunit Rev7, polyubiquitination of PCNA by *Mms2-Ubc13-Rad5* promotes error-free lesion bypass. Inactivation of these two pathways results in a synergistic effect on DNA-damage responses; however, this two-branch DDT model has not been reported in any multicellular organisms.

In order to examine whether *Arabidopsis thaliana* possesses a two-branch DDT system, *rad5a rev3* double mutant plants were created and compared with the corresponding single mutants. Arabidopsis *rad5a* and *rev3* mutations are indeed synergistic with respect to growth inhibition induced by replication-blocking lesions, suggesting that *AtRAD5a* and *AtREV3* are required for error-free and TLS branches of DDT, respectively. Unexpectedly this study reveals three modes of genetic interactions in response to different types of DNA damage, indicating that plant *RAD5* and *REV3*are also involved in DNA damage responses independent of DDT. By comparing with yeast cells, it is apparent that plant TLS is a more frequently utilized means of lesion bypass than error-free DDT. In addition, it was also observed that treatments with the DNA damaging agent methylmethanesulfonate increased the nuclear ploidy level in the double mutant plants.

ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisors Dr. Wei Xiao and Dr. Hong Wang for their continuous support and encouragement during my study. Dr. Xiao and Dr. Wang guided me all through these years and provided me with inspiration and direction for my research.

I would like to thank my committee members Dr. Adrian Cutler and Dr. Hughes Goldie for their valuable feedback and suggestions.

I would like to thank the members of Dr. Xiao's laboratory: Dr. Audesh Bhat, Amanda Lambrecht, Hania Dworaczek, Jia Li, Ke Zhang, Dr. Kamakshi Balakrishnan, Dr. Lindsay Ball, Dr. Landon Pastushok, Michelle Hanna, Michael Biss, Dr. Parker Andersen, Dr. Rui Wen, and Susan Butcher. I would like to thank especially to Dr. Rui Wen, for the discussion and help all through my graduate study. Amanda Lambrecht provided help in the yeast work.

And also, I would like to thank the members of Dr. Wang's laboratory: ShifengQian, Dr. Xianzong Shi, and Yan Chen. Dr. Shi provided help with various tips in cloning and other laboratory techniques. Yan Chen and Shifeng helped me take care of my plants when I was away.

I would like to thank the Departments of Microbiology and Immunology and of Biochemistry, College of Medicine.

Finally, my special thanks would go to my parents and my wife Yuting Luo, for their loving considerations, constant encouragement, and great patience during my graduate studies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

At	*Arabidopsis thaliana*
a.a.	amino acids
AD	activation domain
Ade	adenine
amp	ampicillin
ATP	adenosine 5'-triphosphate
BD	binding domain
bp	base pair
Da	Dalton
dd	double-distilled
DDT	DNA-damage tolerance
DMSO	dimethyl sulfoxide
E1	ubiquitin activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin ligating enzyme
EDTA	Ethylenediaminetetraacetic acid
Gln	glutamine
Glu	glutamic acid
Gly	glycine
GST	Gluthathione-S-Transferase
HRP	Horseradish Peroxidase
h	human
His	histidine
IPTG	isopropyl-β-D-thiogalactopyranoside
K	lysine
LB	Lennox broth
Leu	leucine
MMS	methylmethanesulfonate
MW	molecular weight
nt	nucleotide

OD	optical density
ORF	open reading frame
PBS	Phosphate Buffered Saline
PRR	postreplication repair
PEG	polyethylene glycol
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PVDF	polyvinylidenedifluoride
RING	really interesting new gene
RT	room temperature
SD	synthetic dextrose
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
ss	single strand
TLS	translesion synthesis
UV	ultraviolet
Wt	wild type
YPD	yeast extract-peptone-dextrose
Ub	ubiquitin
Ubc	ubquitin-conjugating enzyme
Uev	biquitin-conjugating enzyme variants

1. LITERATURE REVIEW AND INTRODUCTION

1.1 General Introduction of DNA Repair in the Budding Yeast *Saccharomyces cerevisiae*

Cellular DNA of all organisms is constantly exposed to DNA-damaging agents from endogenous and exogenous sources. The former refers to those created by hydrolytic and oxidative reactions inside the cells. The latter refers to those caused by physical and chemical agents of external sources applied to an organism or cells. The damage created either endogenously or exogenously can modify the primary components of the double helix DNA, for example, the base, sugar and phosphodiester linkage. Due to the essential nature of DNA, the modifications have significant effects on biological processes. DNA replication, which is an essential process for cell division, can be stalled by such structure alternations on DNA strands. In this thesis, exogenous sources of DNA damage will be mainly focused on since several of them were applied experimentally in the project. On the other side, cells develop different mechanisms to survive such DNA damage, by repairing the DNA alternation or proceeding DNA replication in the presence of DNA damage. In this research, the unicellular model organism baker's yeast *S. cerevisiae* and the plant model organism *Arabidopsis thaliana* are used. Different mechanisms of DNA repair in *S. cerevisiae* are discussed below.

1.1.1 Different Sources of DNA Damage

Exogenous DNA-damaging sources include ultraviolent (UV) radiation, γ ray radiation and certain chemical agents that are capable of altering DNA structures. For example, UV radiation causes linkage of two base groups in the same DNA strand; γ ray radiation creates double-strand breaks on the DNA; alkylating agents add a bulky alkyl group onto the DNA bases; and cross-linking agents link two DNA strands together. It is unusual for certain DNA-damaging sources to create only one type of DNA damage. On the other hand, cells possess several

mechanisms working in concert to repair or tolerate different types of DNA damage.

1.1.1.1 UV Radiation

UV radiation is one of the most common DNA-damaging sources. A large number of daytime-living organisms are exposed to extensive solar UV radiation. Not surprisingly, these organisms have developed sophisticated systems to minimize such genotoxic effects since the beginning of evolution. UV irradiation can be divided into three different wavelength groups: UV-A (320 to 400 nm), UV-B (295 to 320 nm) and UV-C (100 to 295 nm). The germicidal lamps, widely used in many laboratories, produce 254 nm UV-C. Since this wavelength is efficiently absorbed by DNA with an absorption peak of 260 nm, but not by proteins. Additionally, it should be pointed out that 254 nm UV-C is not common in natural environment, because UV-C can barely penetrate atmospheric ozone layer. Nevertheless, many lesions in DNA produced by UV-A and UV-B can also be efficiently produced by UV-C.

The two most frequent DNA lesions caused by UV-C radiation are cyclobutanepyrimidine dimer (CPD) and (6-4) photoproduct. CPD is a four-ring structure formed between two adjacent pyrimidines linked covalently. Because of weak bonding of hydrogen with such lesion, this type of structure can perturb the normal DNA structure and cause arrest of DNA replication fork. Among different types of CPDs, the yield of thymine<>thymine (T<>T) CPD is the highest in radiated DNA while that of cytosine<>cytosine (C<>C) CPD is the lowest (Mitchell et al., 1992). Another main lesion caused by UV irradiation is the pyrimidine-pyrimidone (6-4) photoproduct, or (6-4) PP, which links the C6 position of 5' pyrimidine to C4 position of the adjacent 3' pyrimidine. This lesion disturbs DNA double helix since the pyrimidine planes are almost perpendicular within such a lesion. TC and CC (6-4) PP are the two main forms in UV-irradiated DNA. TT (6-4) PP is less frequent and CT (6-4) PP is the least. In

the irradiated DNA by UV-C light, an approximate ratio of 3:1 between CPD and (6-4) PP is observed (Mitchell, 1989).

There are some other lesions caused by UV irradiation. Under anhydrous conditions, 5, 6-dihydro-5-(α-thyminyl)-thymine or so-called "spore photoproduct" can be produced by addition of a methyl group of a thymine to the C5 position of a neighboring thymine. This type of lesion can be largely observed in UV irradiated bacterial spores of *Bacillus subtilis*. About 30% of the thymine can be converted to spore photoproduct in spore DNA after exposure to high dose of UV (Douki, 2003). Pyrimidine hydrate is another type of lesion which arises from the addition of a water molecule across the 5, 6 double bond to form a 5, 6-dihydro-6-hydroxyl derivative. Cytosine hydrate may be the major non-dimer photoproduct of cytosine, which can be dehydrated to yield uracil (Boorstein et al., 1990). The hydrate of 5-methylcytosine may go through deamination to yield thymine hydrate, which can be converted to thymine by dehydration (Vairapandi and Duker, 1994). This conversion may explain UV-induced mutation at methylated cytosine residues.

The interstrand crosslinks may be formed as spore photoproducts since the reaction not only occurs between two adjacent pyrimidines in the same DNA strand, but also between two different DNA strands (Douki et al., 2003). Irradiation with very high doses of UV-C light can cause breakage of the DNA polynucleotide chain (Rosentein, 1983). In cells, however, most strand breaks after UV irradiation due to indirect biological processing, either from interruption of stalled DNA replication forks or as intermediates in the repair of photoproducts (Friedberg et al., 2006).

1.1.1.2 Chemical Agents

A number of chemical agents can cause DNA damage and some of them are used in cancer chemotherapy by inhibiting the proliferation of tumors. The chemical agents can react with DNA in several ways and some representative agents are discussed here.

Alkylating agents are chemicals capable of transferring an alky group to another molecule. They are electrophilic compounds with affinity to nucleophilic centers. These include methylnitrosourea (MNU), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), methyl methanesulfnate (MMS) and ethyl methanesulfonate (EMS). Alkylating agents can be divided into two major subfamilies: mono-functional and bifunctional alkylating agents. The former have only one reactive group which can interact with DNA while the latter have two reactive groups. Thus, the bifunctional agents can interact with DNA at two sites. Many sites in all bases can be alkylated; however, they may have different reactivities. Among them, N7 position of guanine and the N3 position of adenine have the most reactivity (Pegg, 1984; Singer, 1982). For example, after MMS treatment 83% methylation are at N7-guanine and 11% at N3-adenine (Pegg, 1984).

Crosslinking agents are those chemical agents that can react with two different sites in DNA, resulting in either intrastrand or interstrand DNA crosslinks. Intrastrand DNA crosslink refers to the linkage between two sites in the same DNA strand while the interstrand DNA crosslinks occur between two DNA strands. Interstrand DNA crosslink has a very important biological relevance since it can prevent the separation of two DNA strands thus blocking DNA replication and transcription. It is reported that a single unrepaired interstrand DNA crosslink in bacterial or yeast can cause cell death, and 40 interstrand DNA crosslinks are sufficient to kill repair-deficient mammalian cells (Lawley and Phillips, 1996; Magana-Schwencke et al., 1982). It should be noted that those crosslinking agents can not only produce intra- or inter-strand DNA

crosslinks, but also can produce monoadduct in which only base of the DNA was modified with crosslinking agents, as well as protein-DNA crosslinks and glutathione-DNA crosslinks. Those crosslinking agents include nitrous acid, mitomycin (MMC), nitrogen mustard, cisplatin, among which cisplatin is widely used in chemotherapy and particularly effective against testicular cancer. In cells treated with cisplatin, there is only small proportion of interstrand crosslinks, around 1%-2% of total adducts. Most of the lesions are 1, 2-intrastrand crosslink between the N7 positions of two neighboring guanines (Eastman, 1987; Pinto and Lippard, 1985).

In addition to those agents that can directly react with DNA, there is another group of nonpolar chemicals that are chemically inactive, but can be potent mutagens and carcinogens since they can be metabolically activated to a more reactive form. For example, a metabolite of *N*,*N*-dimethyl-4-aminoazobenzene (butter yellow), a potent liver carcinogen in rats, can bind to rat liver proteins, but the compound itself cannot (Miller, 1947). 4-Nitroquinoline 1-oxide (4NQO) also belongs to this group, although its metabolic activation and DNA adduct formation are not entirely understood. It is a quinoline derivative and a tumorigenic compound used in the assessment of the efficacy of diets, drugs, and procedures in the cancer prevention and treatment in animal models. This chemical produces bulky base damage such as adducts at C8 of guanine, the exocyclic N^2 (the exocyclic nitric group at C2 postion) of guanine and N^6 of adenine (Galiegue-Zouitina et al., 1986). The N^2 adduct appears to be the major lesion that accounts for 50-80% of all quinolone based adduct, depending on the superhelicity of the target (Menichini et al., 1989). This type of damage can be repaired principally by nucleotide excision repair (NER), which will be discussed later, like CPD and (6-4) PP in the case of UV irradiation. Thus, 4NQO is often referred to as a "UV mimic agent". 4NQO treatment can also result in the formation of 8-hydroxyguanine, and also lead to significant amount of strand breakages, which probably

indicates the formation of unstable adducts (Galiegue-Zouitina et al., 1985).

1.1.2 Different DNA Repair Pathways in *S. cerevisiae*

DNA alterations can lead to mutagenesis, genome rearrangements and even cell death. To maintain the genomic integrity, a variety of DNA repair mechanisms have been evolved to protect cells from DNA damage. In this thesis, different mechanisms in budding yeast *S.cerevisiae* will be mainly focused, since they are much better understood in yeast than in plants.

1.1.2.1 Direct Reversal

1.1.2.1.1 Direct Reversal of Base Damage

Direct reversal of DNA damage is an efficient way of repairing base damage and often needs a single enzyme to catalyze a single step reaction. Some base damage caused by UV radiation or alkylating agents can be repaired by this direct reversal.

As discussed before, CPD and (6-4) PP are the two major sources of base damages to cells after exposure to UV radiation. One DNA repair mode called enzymatic photoreactivation (EPR), or simply photoreactivation, can directly reverses both CPD and (6-4) PP to pyrimidine monomers. Since this process requires light in particular spectrum of wavelengths, it is called photo-activation. The enzymes that catalyze EPR of CPD and (6-4) PP are called photolyases, which are encoded by one family of genes. This family has close to 30 members that code for proteins with three related but distinct functions: PD-DNA photolyase, (6-4) PP-DNA photolyase and cryptochrome.

In yeast, the gene *PHR* (*PHotoreactivation Repair deficient*) encodes the exclusive PD-DNA photolyase. Purified Phr protein shows a turnover rate of 0.7 CPD monomerized $min^{-1}molecular^{-1}$. It can complement *E.coli phr* mutants defective in EPR (Sancar et al., 1987). It has been estimated that under constitutive conditions there are about 250 to 300 molecules of PD-

DNA photolyase in yeast cells (Sedgwick and Vaughan, 1991).

For a long time, the term EPR had been exclusively associated with the monomerization of CPD in DNA. Another type of enzyme called (6-4) PP-DNA photolyase exists for repairing a major photoproduct (6-4) PP in DNA. The (6-4) PP-DNA photolyase was identified in Drosophila firstly, and has also been detected in some vertebrates and plants (Todo, 1999). However, yeast does not seem to have a homolog. The enzyme from Drosophila can bind to (6-4) PP in DNA with high affinity (Zhao et al., 1997). There is a high degree of conservation at the amino acid level between PD-DNA photolyases and (6-4) PP-DNA photolyases. However, the two types of enzymes have different specificities of substrate, and the structures of their substrate binding sites are presumably different as well (Vande Berg, 1998).

The third class of proteins called cryptochromes have significant amino acid sequence identity and similarity to both PD- and (6-4) PP-DNA photolyases. Surprsingly, these proteins show no EPR activity. Cryptochromes are encoded by a group of plant blue-light receptor genes discovered in Arabidopsis at about the same time as the (6-4) PP-DNA photolyase genes. The CRY1 gene was identified in an Arabidopsis mutant defective in hypocotyl elongation, a process dependent on blue-light-sensing pathway (Ahmad and Cashmore, 1993). CRY2 was also identified and found to be involved in the timing of flowering in response to light (Lin et al., 1998). Research on cryptochromes in mammals has demonstrated that these proteins are also involved in circadian rhythm (Thresher et al., 1998).

1.1.2.1.2 Reversal of Alkylation Damage in DNA

In addition to the repair of two major photoproducts by the one-step enzymatic reaction that directly reverses base damage, there is a similar way of such direct reversal to repair alkylation base damage. These bulky methyl groups in DNA can be removed by a type of

enzymes called DNA alkyltransferases. For example, when cell extracts of *E.coli* were incubated with DNA containing radio labeled O^6-methylguanine, the labeled methyl groups were shown to be associated with O^6-alkylguanine-DNA alkytransferase (O^6-AGTI). It can remove the methyl group from O^6-methylguanine in DNA and transfer it to a cysteine residue in the protein (Olsson and Lindahl, 1980). The enzyme activity has been studied in many eukaryotes, such as yeast, worm, fruit fly, and mammalian cells. A comparison of eight alkyltransferases from bacteria, yeast and mammalian reveals conserved amino acids (Sakumi et al., 1991). In *S. cerevisiae*, the gene encoding O^6-alkylguanine-DNA alkytransferase was designated as MGT1 and identified by functional complementation of the bacterial *ada ogt* double mutant which is defective in the function of alkytransferase (Xiao et al., 1991). Mutants carrying a disruption in the *MGT*1 gene are sensitive to mutagens since they lack the O^6-ATG activity.

1.1.2.2 Base Excision Repair

Base excision repair (BER) a process by which a damaged base is removed and is considered as the most frequent DNA repair mode in nature. In this pathway, a class of DNA repair enzymes called DNA glycosylases excise the damaged base by catalyzing the hydrolysis of the N-glycosyl bonds linking damaged bases to the deoxyribose-phosphate backbone. The initial enzymatic excision during BER forms apurinic/apyrimidinic (AP) sites where a purine or pyrimidine is missing from DNA . The removal of AP sites is accomplished by a second class of BER enzymes called AP endonucleases, which specifically recognize these AP sites in DNA. These endonucleases produce incisions or nicks in DNA by hydrolysis of the phosphodiester bond immediately upstream to the AP site. It also should be noted that some DNA glycosylases have an associated activity called an AP lyase activity, which can cleave the DNA chain downstream to the AP site.

Hydrolysis of phosphodiester bond immediately 5' to an AP site generates a 5' terminal deoxyribose-phosphate residue that can be removed by another class of enzymes, including exonucleases and specific DNA-deoxyribophophodiesterase (dRpase). A single nucleotide gap is thus generated in the DNA. The repair of double-stranded DNA by BER is not completed until the missing nucleotides refilled by DNA synthesis and covalently joined to the parental DNA.

Many DNA glycosylases have been well characterized and they contain a DNA binding domain known as the helix-hairpin-helix (HhH) motif. It contains two α-helices connected by a hairpin turn in a span of about 50 amino acids. Crystal structures of many glycosylases reveal that the overall fold of these proteins is quite conserved. HhH motif forms a deep cleft structure that accepts the nucleotides substrate in the glycosylation reaction (Yamagata et al., 1996). The HhH superfamily in *E. coli* contains different members classified by their basic function: AlkA for alkyladenine-DNA glycosylase, Mpg II for N-methypurine-DNA glycosylase II, MutY/Mig for A/G-specific adenine glycosylase/mismatch glycoslase, Nth for endonuclease III, OggI for 8-oxoG-DNA glycosylase I and OggII for 8-oxoG-DNA glycosylase II (Denver et al., 2003). These enzymes excise different types of modified bases, demonstrating the versatility of the HhH protein fold and its adaptation to the repair of different types of damage.

1.1.2.3 Nucleotide Excision Repair

Like BER, NER is also a multistep process. It leads to the formation of gaps in the DNA that must be filled by repairing synthesis and covalently sealed by DNA ligation. In contrast to BER, which excises chemically modified bases, NER is a totally distinct repair pathway in which a short oligonucleotide containing the DNA lesion is excised. Additionally, BER mainly repairs those chemically modified bases, whereas NER mainly works on the major photoproducts in DNA produced by UV radiation. This process is also referred as dark repair to denote its

independence of photoreactivation.

S.cerevisiae is well characterized genetically with respect to its response to agents that cause DNA damage. As well, for the study of NER, the amenability of this organism to genetic and biochemical studies has served well for the discovery of many useful mutants. For example, over 30 *RAD* loci (for "radiation sensitive") that confer resistance to UV and ionizing radiation have been identified and classified into three epistasis groups in terms of different radiation sensitivity.

Epistatic interactions are operationally defined by the use of mutant strains. If a mutant strain carrying mutations in two different genetic loci (A and B) displays a phenotype, for example, sensitivity to UV radiation, that is quantitatively the same as the strain carrying a single mutation (A), the mutation A is said to be epistatic to mutation B. In contrast, if combination of the two mutations shows additive effects, the two corresponding genes belong to different epistatic groups. The simplest interpretation of epistatic interactions between different mutations is that the genes they represent are involved in sequential steps of a multistep biochemical pathway or that they encode components of a complex. However, such divisions are not always the case.

The inactivation of genes in the *RAD3* epistasis group results in an increased sensitivity to UV radiation and many chemicals that induce bulky base adducts in DNA, and majority of these genes encodes proteins involved in NER. The *RAD3* epistasis group genes include *RAD1*, *RAD2*, *RAD3*, *RAD4*, *RAD10*, *RAD14*, *RAD23* and *RAD25* (Friedberg, 2006). The representative gene of this group, *RAD3* is essential for viability in yeast cells in the absence of DNA damage. The essential function of *RAD3* is related to its role in the RNAPII transcription. More specifically, *RAD3* is the yeast ortholog of human *XPD* or ERCC2 gene that encodes a

component of the transcription factor TFIIH, which functions to open the DNA helix during NER (Douziech et al., 2000). Most subunits of the yeast core TFIIH complex, including Rad3, Ssl2 (Rad25), Ssl1, Tfb1, Tfb2, Tfb3, and Tfb4, are required for both NER and RNAPII transcription.

1.1.2.4 Homologous Recombination Repair

Double strand breaks (DSBs) are conveniently induced by ionizing radiations (X rays or γ rays) (IR) or radiomimetic chemicals such as bleomycin. DSB can also be produced as intermediate products in the DNA repair processes. One major pathway of DSB repair involves a mechanism referred to as homologous recombination (HR) that utilizes similar or identical DNA sequences. A series of events are initiated by unidirectional 5' to 3' DNA degradation following DSB. The resulting 3' single-stranded DNA (ssDNA) ends can invade into homologous double-stranded DNA sequence and displace the original pairing strand, resulting in a heteroduplex. Homologous DNA may be located on the sister chromatid, different locus or even on different chromosomes, resulting in interchromatid, intrachromosomal or interchromosomal recombination, respectively. Two typical Holliday junctions are generated after strand invasion and DNA is synthesized on the invading ssDNA using homologous sequences as a template. Holliday structures can be resolved by coordinated single-strand scissions and rejoining, orchestrated by a specialized protein complex called resolvase.

In budding yeast, most of the genes required for homologous recombination, including *RAD50, RAD51, RAD52, RAD54, RAD55, RAD56, RAD57, RAD59, XRS2* and *MRE11*, fall into the *RAD52* epistasis group (Friedberg, 2006). The *rad52* mutant is characterized by a broad range of defects in mitotic and meiotic recombination, in the repair of DSB induced by IR or chemicals. Rad52 can act together with Rad51 as a ssDNA-binding protein and promote

annealing of the complementary ssDNA (Mortensen et al., 1996).

1.1.2.5 DNA Damage Tolerance

In addition to the highly conserved DNA repair pathways discussed above, all living organisms have evolved means to ensure DNA synthesis in the presence of DNA damage. These schemes were originally termed DNA postreplication repair (PRR) due to observations of transient shorten nascent DNA structures following S phase in response to DNA damage.

One mechanism in dealing with the DNA lesion is to bypass it, without correcting the replication-blocking lesion. Perhaps it is more beneficial for the organism to tolerate DNA damage rather than to allow the replication fork to collapse. Unlike other DNA repair mechanisms, this pathway does not actually remove a damaged lesion and for that reason it is called DNA damage tolerance (DDT). In eukaryotes, DDT is evolutionarily conserved and consisted of two alternative pathways. One is translesion DNA synthesis (TLS), which is considered error prone because utilizing low fidelity polymerase to bypass DNA damage results in the increased mutability. Another is error-free bypass, with a high degree of fidelity. In the following, the DDT in eukaryotes will be described in more details.

1.2 DNA Damage Tolerance in *S. cerevisiae*

Treatment with many DNA-damaging agents results in an increasing mutation rate in *S. cerevisiae*. Some of these mutants such as *rad6* or *rad18*, which exhibit severely reduced mutability, belong to the collection of previously isolated *rad* mutants. These mutants are highly sensitive to UV, ionizing radiation and other DNA-damaging agents. Additionally, there are other mutants in the same epistasis group, such as *rad5*, showing increased sensitivity but no reduced mutation rate (Kunz and Haynes, 1981).

Genetic screens for mutants that have reduced reverse mutation frequencies resulted in

the isolation of a class of mutants designated *rev* (for "defective mutation reversion") (Lemontt, 1971a, b). The above *RAD* and *REV* genes belong to the *RAD6* epistasis group that contains *RAD5 (REV2)*, *RAD6*, *RAD18*, *RAD30*, *UBC13*, *MMS2*, *REV1*, *REV3* and *REV7* (Friedberg et al., 2006).

1.2.1 Translesion DNA synthesis in *S. cerevisiae*

1.2.1.1 DNA Polymerase ζ

The *S. cerevisiae REV3* gene is of special interest because the *rev3* mutant has a dramatic reduction in damage-induced and spontaneous mutation rates. *REV3* encodes a protein of about 173 kDa containing regions homologous to known DNA polymerases, in particular *Epstein-Barr* virus and herpes simplex virus DNA polymerases (Morrison et al., 1989). Between Rev3 and the *Epstein-Barr* virus DNA polymerase, there is 23.9% amino acid identity and 60% similarity. There are also general structural similarities between Rev3 protein of the budding yeast and human B family DNA polymerases, such as two zinc finger-like DNA-binding regions encoded by a cysteine-rich C-terminal domain. Since the *rev3* mutant of *S. cerevisiae* is viable, this putative DNA polymerase is considered nonessential. The transcription of *REV3* is neither cell cycle regulated nor UV radiation inducible (Singhal et al., 1992).

REV7 deletion in budding yeast results in a similar effect in damage-induced mutability as *REV3* deletion (Lawrence et al., 1985a; Lawrence et al., 1985b). Rev7 can interact with Rev3, and purified Rev3 protein only exhibits significant DNA polymerase activity when in a complex with Rev7 (Nelson et al., 1996b). Together, these two proteins constitute DNA polymerase ζ (Polζ). This polymerase has low processivity *in vitro*, since 50% of the protein dissociates from the template after extending the primer by only 1 to 3 nucleotides. However, the most remarkable activity of Polζ is to bypass lesions induced by UV radiation. If a TT dimer is

present in the template, the efficiency of DNA synthesis past the dimer is about 10%, which is 10-fold higher than the efficiency of a typical replicative polymerase such as DNA Pol α (Nelson et al., 1996b). It should be noted that this CPD bypass activity of Polζ is still considered weak *in vitro*, which could be enhanced through collaboration with other proteins *in vivo*.

The human homolog of *S. cerevisiae REV3* encoding the catalytic subunit of Polζ was also isolated (Gibbs et al., 1998; Xiao et al., 1998). Interestingly, the predicted DNA polymerase region is conserved and an N-terminal stretch shows sequence similarity to the yeast enzyme, but there is a large region between residues of 333 and 1888 with no counterpart in the *S. cerevisiae* Rev3 sequence (Gibbs et al., 2000). Very little is known about this stretch of sequence. Human fibroblasts have been transformed with antisense RNA constructs, and 4- to 6-fold reduction of UV-induced mutability was achieved without any influence on survival (Diaz et al., 2003). These suggest that human *rev3* participates in UV radiation-induced mutagenesis, similar to the role of Rev3 in *S. cerevisiae*.

1.2.1.2 Rev1

S. cerevisiae REV1 also plays a role in TLS. This nonessential gene encodes a protein of 112 kDa containing an internal stretch of 152 residues with 25% identity to the *E.coli* UmuC protein (Larimer et al., 1989), which is a component of polymerase IV (Wagner et al., 1999). However, unlike *umuC* in *E.coli*, *REV1* expression is not induced by DNA-damaging agents in *S. cerevisiae*. The Rev1 structure and functions appear to be highly conserved in higher eukaryotes. An decrease in UV-induced mutagenesis was observed in cultured human cells with downregulation of *REV1* expression (Gibbs et al., 2000).

Purified *S. cerevisiae* Rev1 and its human homolog (Rev1L) show deoxycytidyltransferase (dCMP transferase) activity that transfers dCMP from dCTP to the 3'

end of a DNA primer (Lawrence, 2002; Nelson et al., 1996a). This reaction is template dependent and occurs preferentially opposite template G, U or an AP site. The resulting terminus, for example, C opposite AP site, cannot serve as a primer for replicative polymerases but can be extended by Pol ζ. Human and mouse Rev1 interacts stably with the Pol ζ via its subunit Rev7 (Guo et al., 2003; Murakumo et al., 2001). However, it has not been demonstrated whether this Rev1- Polζ interaction specifically facilitates primer extension following base insertion by Rev1 protein. In *S. cerevisiae*, UV radiation-induced mutability is reduced if *REV1* is deleted. However, this reduction of mutability cannot be explained by its dCMP transferase activity (Zhang et al., 2002). Indeed, analysis of site-specific mutations confirms that the Rev1 enzymatic activity is not essential for TLS, but its BRCA1 C-terminal (BRCT) domain (Otsuka et al., 2005) or a polymerase-associated domain (PAD) (Acharya et al., 2005) is required for the protein interaction. The C-terminal 100 amino acids of human Rev1 are sufficient to interact with Rev7 and other Y-family TLS polymerases, implying that Rev1 may play a scaffold role in TLS (Guo et al., 2003).

1.2.1.3 DNA Polymerase η

Polymerase η (Pol η) in *S. cerevisiae*is is encoded by *RAD30*. The inactivation of *RAD30* or its homolog of mammalian Xeroderma Pigmentosum Variant (*XPV*) gene leads to an increased susceptibility to UV-induced DNA damage (Johnson et al., 1999a; McDonald et al., 1997). Polη is able to correctly incorporate two nucleotides AA opposite the TT dimers in the template DNA with a fidelity and efficiency as good as when passing normal T residues (Johnson et al., 1999b). This bypass is hence considered as error-free. However, for the other types of lesions such as other types of CPD and TT (6-4) photoproducts, Pol η has a lower rate of incorporate nucleotides and lower fidelity. Hence, Polη seems to be highly specialized and is the only known "error-free"

polymerase when bypassing TT dimer.

Pol η (Rad30) belongs to the Y family of DNA polymerases with primary sequence similarity to each other but not to known replicative polymerases (Ohmori et al., 2001). This family is formerly known as the UmuC/DinB/Rev1/Rad30 superfamily. These members have different distribution among the kingdoms of life. The UmuC family is only found in bacteria, the Rev1 and Rad30 are found only in eukaryotes, and DinB family can be found in both prokaryotes and eukaryotes.

1.2.2 The Rad6-Rad18 Complex and Ubiquitination

1.2.2.1 Rad6 and Ubiquitination

Mutants defective in the *RAD6* gene of *S. cerevisiae* are highly sensitive to a variety of DNA-damaging agents, including UV radiation, γ-rays, alkylating agents, and crosslinking agents. Interestingly, *rad6* mutants show reduced mutability after treatment with many DNA-damaging agents. The characterization of different *rad6* alleles of *rad6* mutants indicates multifunctional roles for the Rad6 protein. For example, *rad6-1* or *rad6-3* alleles both show sensitivity to those DNA-damaging agents. *rad6-1/rad6-1*but not *rad6-3/rad6-3* diploids are additionally deficient in sporulation (Montelone et al., 1981).

Since Rad6 protein is involved in protein ubiquitination, I would like to first introduce the concept of protein ubiquitination. Ubiquitin (Ub) is a small protein, ubiquitously present in all eukaryotic organisms from unicellular yeast to human. Ub contains 76 amino acids, with a molecular mass of 8.5 kD. Ub can be covalently attached to itself or to a specific target protein, a process called protein ubiquitination. Ubiquitination typically consists of three steps in forming an isopeptide bond (Pickart, 2001). First, Ub is activated by an Ub-activating enzyme (Uba or E1) in the presence of ATP to form an E1-Ubthiolester with high energy. Then the activated Ub is

transferred to a specific member of the Ub-conjugating enzyme (Ubc or E2) family to form an E2-Ub thiolester. Finally, the E2-Ub donates its Ub to the target protein either by itself or through an Ub ligase (E3). The substrate specificity is generally believed to be conferred by E3 ligases.

Substrate proteins can be ubiquitinated in different ways. In monoubiquitination, only one Ub is attached to a Lys residue of the target protein. In multiubiquitination, two or more Lys residues of a protein are modified each by one Ub molecule. In polyubiquitination, a polyubiquitination chain is formed in modifying a protein (Haglund and Dikic, 2005; Pickart, 2001). Ub contains seven Lys residues, Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63. The polyubiquitination chain can be formed through the C-terminal Gly76 of an Ub and Lys48 of the next Ub, which is called K48-linked polyubiquitination (Pickart and Fushman, 2004). It is known that K48-linked polyubiquitinated proteins are targeted and degraded by 26S proteasome. This process of ubiquitin-mediated proteolysis regulates the levels and dynamics of many important proteins and is important in cellular processes (Pickart and Fushman, 2004). Rather than the conventional K48-linkage, a polyubiquitin chain can also be linked through Gly76 of aUb to Lys63 of the next Ub, which is called K63-linked polyubiquitination. In contrast to the function in proteasomal protein degradation of target proteins by the K48-linked polyubiquitination, K63-linked polyubiquitination plays diverse non-proteolytic functions (Pickart, 2001). So far, Ubc13 is the only known E2 enzyme capable of catalyzing K63-linked polyubiquitination, which requires an Ubc/E2 variant (Uev) as a co-factor (Hofmann and Pickart, 2001; McKenna et al., 2001). Uev proteins are similar to Ubcs, but lack of a critical Cys residue in the active site. K63-linked polyubiquitination is known to play signaling roles in several processes, including inflammatory response, protein trafficking, ribosomal protein synthesis, and

DNA damage tolerance (Pickart and Fushman, 2004).

RAD6 encodes a protein of 19.7 kDa containing a highly acidic C terminus of 13 consecutive Asp residues. The protein has been shown to be one of several E2 enzymes in the *S. cerevisiae* ubiquitination system. It can catalyze the transfer of activated ubiquitin to substrate proteins such as histones H2A and H2B *in vitro* and *in vivo* (Jentsch et al., 1987; Sung et al., 1988). In the budding yeast, there are other mutants that share some similarity with the *rad6* mutants defective in ubiquitin transaction. Inactivation of the *UBI4* gene (encoding a single polypeptide consisting of multiple ubiquitin moieties) or the *UBR1* gene (encoding a yeast E3 enzyme) results in defective sporulation and additionally causes abnormal sensitivity to several stress conditions (Bartel et al., 1990; Finley et al., 1987). Although Rad6 shares sequence similarities with other E2 enzymes such as Cdc34, decreased mutation frequency following exposure to DNA-damaging agents is unique to *rad6* mutants among genes encoding E2 enzymes.

Orthologs of *RAD6* genes have been identified in many eukaryotic organisms other than *S. cerevisiae*. Single copy genes with extensive amino acid sequence similarity have been characterized in *S. pombe* (*rhp6*$^{+}$) (Reynolds et al., 1990) and *Drosophila melanogaster* (*UbcD6/Dhr6*) (Koken et al., 1991a; Reynolds et al., 1990). In human, two *RAD6* genes (*UBE2A/RAD6A/HHR6A* and *UBE2B/RAD6B/HHR6B*), whose products have 95% amino acid identity with yeast Rad6, have been identified (Koken et al., 1991b). While deletion of the *Hr6b* gene alone in mouse results in no obvious phenotype except male infertility (Roest et al., 1996), deletion of both mouse homologs *Hr6a* and *Hr6b* causes death (Roest et al., 2004). This phenotype may correlate to the phenotype of defective sporulation in *S. cerevisiae*. The phenotype may be explained by the inability to ubiquitinated histone H2A or to properly form

synaptonemal complex structures during meiotic prophase (Baarends et al., 2003).

An acidic tail is unique to the *S. cerevisiae* Rad6 protein and required for polyubiqutination of histone H2B in *in vitro* and *in vivo* (Robzyk et al., 2000; Sung et al., 1988). However, deletion of the entire C-terminal tail of Rad6 has no effect on its role in DNA repair and UV-induced mutagenesis, but affects only sporulation (Morrison et al., 1988). The N-terminal 15 amino-acid sequences of all Rad6 homologs are nearly identical. Deletion of the first 9 amino acids of Rad6 (Rad6$\Delta_{1\text{-}9}$) abolishes sporulation, reduces cell survival after UV treatment, but surprisingly increases spontaneous and UV-induced mutagenesis (Watkins et al., 1993). Furthermore, the N-terminus of Rad6 is also required for N-end rule protein degradation. The full-length Rad6 can interact with the E3 enzyme Ubr1 which is required in N-end rule protein degradation, while the Rad6$\Delta_{1\text{-}9}$ protein is unable to interact with Ubr1 (Watkins et al., 1993).

1.2.2.2 Rad18

Rad6 appears to be a multi-functional E2 with different partners. In addition to Ubr1, Rad6 is known to form a table complex with Rad18, and this complex displays Ub conjugation, ssDNA-binding and ATPase activity (Bailly et al., 1994; Bailly et al., 1997). Like *rad6*, the *rad18* mutant is extremely sensitive to UV and a variety of DNA-damaging agents, and displays a mutator phenotype under normal conditions (Jones et al., 1988). However, in contrast to *rad6*, *rad18* displays a signature spontaneous GC-to-TA mutation increase and does not display slow growth and sporulation defects (Kunz et al., 1991). This indicates that the DDT activity of Rad6 is achieved through interaction with Rad18 (Broomfield et al., 2001).

The 55.5-kDa Rad18 protein has several important features. A consensus nucleotide-binding RING finger domain has been identified at the N terminus. The RING finger domain contains four pairs of zinc ligands (normally cysteines), which coordinately bind two zinc ions,

and is a signature feature of this family of E3 enzymes (Freemont et al., 1991). Their function is not only to recruit E2 enzymes to the vicinity of the substrates but also to mediate the transfer of ubiquitin from the E2 enzyme to the substrates (Deshaies and Joazeiro, 2009).

Homologs to Rad18 have been identified in lower and higher eukaryotes. Mouse and human Rad18 are able to interact with both HR6A and HR6B protein (Tateishi et al., 2000; Xin et al., 2000). As in budding yeast, disruption of *RAD18* in human cells or in DT40 chicken lymphocyte results in sensitivity to a variety of DNA-damaging agents (Yamashita et al., 2002). Rad18 may have a function as a PAD protein that loads Rad6 to the sites of DNA damage since it can bind to single-stranded DNA (Bailly et al., 1994; Bailly et al., 1997). Similarly, the human *RAD6B* homolog is recruited to chromatin in response to DNA damage (Lyakhovich and Shekhar, 2004).

1.2.3 Error-free Bypass in *S. cerevisiae*

1.2.3.1 Mms2-Ubc13 complex

Since Rad6 and Rad18 are involved in the error-free DNA repair pathway, the fact that *rad6* and *rad18* mutants are much more sensitive to DNA-damaging agents compared to *rev3* and *rad30* mutants suggests that a significant fraction of repair activities are error free. Another mutant called *mms2* (Methyl MethaneSulfonate sensitivity 2) was identified and has epistatic interaction with *rad6* or *rad18* mutations but nonepistatical interaction with *rev3* or *rad30* mutations (Broomfield et al., 2001). The *MMS2* gene belongs to an evolutionarily conserved family encoding ubiquitin-conjugating enzyme variant (UEV) proteins. These proteins interact partner with the E2 enzyme partner Ubc13 and catalyze an unconventional polyubiquitination reaction (Hofmann and Pickart, 1999). Instead of forming a polyubiquitin chain linked through Lys48 of ubiquitin, the polyubiquitin chain formed by Ubc13-Uev complex is through Lys63.

Lys63-linked protein ubiquitination appears to play an important signaling role in DNA repair. Indeed, budding yeast with ubiquitin K63R mutation is sensitive to DNA-damaging agents (Spence et al., 1995). So far, Ubc13 is the only E2 found to catalyze K63-linked polyubiquitination.

Ubc13 and Uev are highly conserved in eukaryote kingdom (Brown et al., 2002). In conditional knockout mouse, somatic deletion of the *Ubc13* gene causes severe loss of multi lineages of immune cells, which is associated with profound atrophy of the thymus and bone marrow, indicating that Ubc13 is essential for the survival and has an important function in mediating hematopoiesis (Wu et al., 2009). As a partner of Ubc13 catalyzing Lys63-linked polyubiquitination, it is interesting to observe that usually there are more Uev proteins than Ubc13 proteins in higher eukaryotes, suggesting that the Uev proteins may have evolved to increase diversity and selectivity of Ub conjugating. Indeed, two mammalian homologues of budding yeast Mms2, Mms2 and Uev1, share more than 90% amino acid sequence identity to each other in their core domain and both can interact with Ubc13 to promote ubiquitination *in vitro*. However, they are involved in different biological activities *in vivo*. Mms2 is required for Ubc13-dependent DNA damage response whereas Uev1A functions in Ubc13-dependent NF-κB activation (Andersen et al., 2005).

Ubc13-Mms2 complex is normally cytosolic, but they are redistributed to the nucleus when there is DNA damage (Ulrich and Jentsch, 2000). This redistribution may suggest that there is a cross talk between this specialized ubiquitination machinery and other members of the *RAD6* epistasis group, which apparently bring the *Ubc13-Mms2* complex to sites of damage. The main contact is made through Rad5, another member of the error-free subpathway in the *RAD6* group.

1.2.3.2 Rad5

rad5 mutants were isolated among a group of reversion-deficient mutants and called *rev2* mutants originally. *Rad5* belongs to the Swi/Snf superfamily of ATPase proteins with a DNA helicase domain. The DNA helicase domain is located to the C-terminal half of the protein and a RING domain is present in its center. However, the DNA helicase activity has not been demonstrated in canonical helicase assays (Johnson et al., 1994).

Rad5 interacts with Ubc13 with its RING figure domain. Rad5 also forms contacts with Rad18. There is evidence for homomeric (Rad5-Rad5 and Rad18-Rad18) interactions of each RING finger protein that may compete with heteromeric (Rad5-Rad18 and Rad5-Ubc13) interaction of the same binding site (Ulrich and Jentsch, 2000). Rad5 thus mediates the contact between Ubc13-Mms2 and Rad6-Rad18 complex that is targeted to single-stranded DNA. In addition, Rad5 is considered as multi-functional protein. It has been reported to promote instability of simple repetitive sequences (Johnson et al., 1992) and to inhibit non-homologous end-joining of DSBs (Ahne et al., 1997). Moreover, Rad5 is involved in double-strand break repair independent of its ubiquitination activity (Chen et al., 2005).

Two human homologs of Rad5, HLTF and SHPRH, have been identified (Motegi et al., 2008; Motegi et al., 2006; Unk et al., 2008; Unk et al., 2006). In addition to the helicase and RING finger domains, yeast Rad5 and human HLTF have a HIRAN domain, but SHPRH does not. Although the biological function of HIRAN domain remains unknown, based on in silico analysis, it has been described as a DNA-binding domain for recognizing damaged DNA or a stalled replication fork (Iyer et al., 2006). The finding of monoubiquitinated PCNA and Rad18 interacting with Rad5 through N-terminal half of the Rad5 HIRAN domain suggests that this domain may function as a platform for protein-protein interactions (Carlile et al., 2009; Ulrich

and Jentsch, 2000). SHPRH has a PHD domain absent in Rad5 and HLTF. The PHD domain has been reported to bind histones and is involved in protein-proteins interaction. The structural difference between SHPRH and HLFT may indicate that these two proteins work in distinct biological processes. Consistent with this notion, a very recent study shows these two proteins have different functions in response to UV and MMS treatments (Lin et al., 2011). Moreover, HLTF and SHPRH are not essential for survival and PCNA polyubiquitination (Krijger et al., 2011), indicating the existence of an alternative E3 ligase.

1.2.4 Sequential Modification of PCNA

PCNA (Proliferating Cell Nuclear Antigen) encoded by *POL30* in budding yeast forms a ring-shaped homotrimer that circles the DNA and operates as a scaffold. The inner ring of PCNA is positively charged because PCNA is rich in lysine and arginine residues. Thus, having positive charges allow for the effective encircling around the negatively charged duplex DNA. PCNA is a DNA polymerase processivity factor. It assembles a number of related proteins required for DNA unwinding and synthesis, cell cycle progression and chromatin structure maintenance (Moldovan et al., 2007).The isolation and characterization of the *pol30-46* allele led to a conclusion that PCNA is involved in DDT. Genetic interaction was investigated between *pol30-46* and other alleles in the *RAD6* epitasis group. *pol30-46* is epistatic to *rad6* and *rad18*, but has a synergistic effect with *rev3*. The *pol30-46* mutant is normal in UV-induced mutagenesis and DNA synthesis, but displays significantly reduced PRR activity via the alkaline sedimentation assay (Torres-Ramos et al., 1996).

PCNA ubiquitination can be induced by different types of DNA-damaging agents, replication stresses or spontaneous DNA damage. Different covalent ubiquitination of PCNA, either monoubiquitination or polyubiquitination, determines which tolerance pathway will be

utilized (Zhang et al., 2011). PCNA can be monoubiquitinated on the Lys164 residue and this reaction is catalyzed by the Rad6-Rad18 complex (Hoege et al., 2002). Purified human Rad18 and Rad6B are able to efficiently monoubiquitinate PCNA *in vitro* (Watanabe et al., 2004). In addition, PCNA monoubiquitination appears to only happen at stalled replication forks because it is limited to PCNA loaded onto DNA by replication factor C (Garg and Burgers, 2005). Polyubiquitinated PCNA was also observed upon DNA damage in cells. This modification is also at the Lys164 residue, which is linked through the Lys63 Ub chain, and requires functions of *MMS2*, *UBC13*and *RAD*5 (Hoege et al., 2002). Hence, it is conceivable that the two E2-E3 complexes Rad6-Rad18 and Mms2-Ubc13-Rad5 sequentially ubiquitinate PCNA. It should be noted that the identical residue of PCNA can be sumoylated by another E2-E3 complex Ubc9-Siz1 (Hoege et al., 2002; Stelter and Ulrich, 2003). So far, the model about the role of PCNA in DDT is well established in *S. cerevisiae*. In response to DNA damage, PCNA can be monoubiquitinated by the Rad6-Rad18 complex. The monoubiquitinated PCNA promotes either error-free or error-prone TLS, which requires Pol η or Pol ζ, respectively. Furthermore, monoubiquitinated PCNA can be polyubiquitinated by the Mms2-Ubc13-Rad5 complex, which promotes error-free DDT (Andersen et al., 2008; Zhang et al., 2011) (Fig. 1.1).

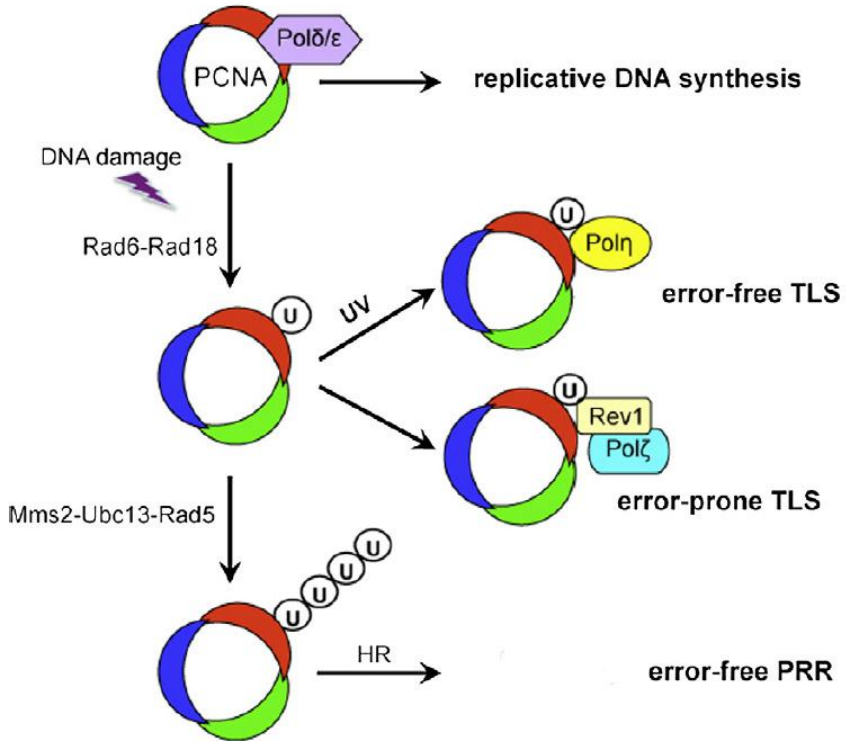


Figure 1.1 Roles of PCNA in replication and DNA-damage tolerance in yeast. PCNA is involved in genomic DNA synthesis as a loading scaffold for the DNA replication. Upon DNAdamage, the Rad6-Rad18 ubiquitination complex mediates at least three different lesion bypass pathways: error-prone TLS mediated by Rev1 and Polζ, error-free TLS mediated by Polη, and error-free bypass mediated by Mms2-Ubc13-Rad5. All these pathways appear to require ubiquinated PCNA. This figure is from (Zhang et al., 2011) with permission.

1.3 DNA Damage Tolerance in *Arabidopsis thaliana*

Currently *Arabidopsis thaliana* is the most widely used model for plant molecular biology and genetics studies. Since it will be used in this study, the discussion will focus on Arabidopsis. There are several advantages of using *A. thaliana*. One is its small genome, whose entire sequence has been determined in 2000 (AGI, 2000). It has a very rapid life cycle of about 6 weeks, and can be efficiently transformed utilizing *Agrobacterium tumefaciens*. Also, a large number of T-DNA insertion mutant lines and genomic resources are available (e.g. from The

Arabidopsis Biological Resources Center). Specifically relevant to this study, Arabidopsis is an excellent multicellular model organism to study genes involved in genome stability because it is more tolerant to genome instability than mammalian models such as mice. In mice, embryonic lethality has been reported in many cases when genes related to DNA repair are deleted (Wu et al., 2009).

Being sessile, plants have to tolerate various harsh conditions such as excessive sunlight radiation, chemical mutagens, fungal toxins, temperature and water stresses. Therefore, plants are expected to have an efficient system of preventing DNA damage or repairing the damage in order to maintain genome stability. There can be serious consequences for plants when genes that function in DNA repair pathways are interrupted, for instance, increased sensitivity to UV light, rapid accumulation of mutations and reduced genome stability (Hoffman et al., 2004; Ries et al., 2000; Sakamoto et al., 2003). It appears that major pathways known in yeast and/or animals including photoreactivation, nucleotide excision repair, and DDT are conserved in plants (Britt, 1999; Hays, 2002). For instance, most of the genes involved in DDT have been found in Arabidopsis (Table 1.1). Only pathways related to DDT will be discussed in this thesis.

Table 1.1 Summary of genes involved in DDT from budding yeast and Arabidopsis

Saccharomyces cerevisiae	*Arabidopsis thaliana*	Locus tags	References
PCNA	*AtPCNA-1*	At1g07370	Anderson et al., 2008
	AtPCNA-2	At2g29250	Anderson et al., 2008
RAD6	*AtUBC1*	At1g14400	Cao et al., 2008 Gu et al., 2009
	AtUBC2	At2g02760	Zwirn et al., 1997 Cao et al., 2008 Gu et al., 2009
	AtUBC3	At5g62540	Cao et al., 2008 Gu et al., 2009
RAD18	Not found		
RAD30	*AtPOLH*	At5g44740	Anderson et al., 2008
REV1	*AtREV1*	At5g44750	Takahashi et al., 2005
REV3	*AtREV3*	At1g67500	Sakamoto et al., 2003
REV7	*AtREV7*	At1g16590	Takahashi et al., 2005
UBC13	*AtUBC13A*	At1g78870	Wen et al., 2006
	AtUBC13B	At1g16890	Wen et al., 2006
MMS2	*AtUEV1A*	At1g23260	Wen et al., 2008
	AtUEV1B	At1g70660	Wen et al., 2008
	AtUEV1C	At2g36060	Wen et al., 2008
	AtUEV1D	At3g52560	Wen et al., 2008
RAD5	*AtRAD5a*	At5g22750	Chen et al., 2008
	AtRAD5b	At5g43530	Chen et al., 2008

1.3.1 Translesion DNA Synthesis in Arabidopsis

In the TLS branch, genes encoding Rev3 (Sakamoto et al., 2003), Rev7, Rev1 (Takahashi et al., 2005), and Polη (Anderson et al., 2008; Curtis and Hays, 2007) have been reported (Table 1.1). Inactivation of these genes causes various extents of sensitivity to UV as well as other DNA-damaging agents. *AtREV3* was identified by mapping an Arabidopsis mutation that confers mild sensitivity to UV-B exposure. The predicted AtRev3 has six C-terminal B family DNA polymerase motifs, and sequences from motifs I-III have perfectly conserved active sites for a

role in DNA replication. The conservation of these domains is significant because this is the region through which yeast Rev3 binds to Rev1(Acharya et al., 2005). Surprisingly, the Rev3 sequences required for interaction with Rev7 in yeast or human cells are poorly conserved in AtRev3. However, two C4 zinc-binding domains conserved at the C-terminus of yeast and human Rev3 proteins are also present in AtRev3. These observations suggest a conserved role for AtRev3 in TLS.

Arabidopsis Rev7 or Rev1 homologues have also been isolated (Table 1.1). AtRev7 contains a region homologous to human Rev7 from positions 21 to 155. This region is responsible for the interaction between Rev3 and Rev1 (Murakumo et al., 2001), suggesting that AtRev7 interacts with AtRev3 and AtRev1.

Rev1 homologues normally have five polymerase domains found in Y family polymerases, and several regions involved in protein interaction. BRCT domain is required for the interaction with PCNA and TLS to pass T-T (6-4) photoproducts (Guo et al., 2006a). Monoubiquitination of PCNA enhances binding by Rev1 through two C-terminal Ub-binding motifs necessary for TLS and damage-induced mutagenesis (Guo et al., 2006b). The PAD of yeast Rev1 binds to Rev7, whereas mouse and human Rev1 interacts with Rev7, as well as Polη and Polκ, via its C-terminal 100 amino acids (Guo et al., 2003; Murakumo et al., 2001). All of these domains are conserved in the predicted AtRev1, suggesting common functions.

Arabidopsis *POLH* encodes a Polη homologue (AtPolη) (Table 1.1). The predicted AtPolη has five Y family polymerase domains essential for DNA synthesis, and two conserved forms of the C-terminal PIP box present in human and yeast Polη. However, AtPolη lacks a UBZ domain present in the C-terminal region of human and yeast Polη proteins, which is required for enhanced binding to monoubiquitinated PCNA (Bienko et al., 2005). Expression of the *AtPOLH*

in a yeast *RAD30* (*RAD30* encodes Polη) deletion mutant could not complement the UV sensitivity of the mutant, but co-expression of *POLH* and *AtPCNA2* restored normal UV resistance in the *rad30* mutant. This complementation was abolished by mutating residues in of the highly conserved polymerase domains in AtPolη (Anderson et al., 2008).

The genetic interactions between these TLS polyermases and their activities in UV radiation induced somatic mutations have been demonstrated in Arabidopsis (Nakagawa et al., 2011). The mutation frequency in *rev3* and *rev1* mutants decreased compared to the wild-type, whereas the mutation frequency in *polh* mutant increased. These observations are consistent with the error-prone TLS of Polζ and Rev1, and error-free TLS of Polη in budding yeast and mammalian cells. Mutation frequency of the *rev1 rev3* double mutant was identical to the *rev1* single mutant, which supports that Polζ and Rev1 work cooperatively to bypass DNA damage. The relatively high mutation frequency in *polh* mutant can be suppressed by *rev3* mutant, suggesting that Polζ is responsible for the high mutation frequency when Polη is not available. In contrast, the finding that mutation frequency of *rev3* single mutant is not lower than that of *rev3 polh* double mutant indicates that Polη cannot complement error-prone pathway when Polζ is unavailable.

1.3.2 Error-free Bypass in Arabidopsis

Genes involved in error-free bypass of DDT have also been found and characterized from Arabidopsis (Table 1.1). Three predicted proteins AtUbc1, AtUbc2 and AtUbc3 were found as Rad6 homologues and all of them contain the conserved cysteine at position 88, which is essential for ubiquitin-conjugating activity (Kraft et al., 2005). Through an *in vitro* ubiquitination assay, AtUbc1-AtUbc3 are able to conjugate ubiquitin to target proteins. However, it is unknown whether they contribute to PCNA ubiquitination in response to DNA damage. Expression of

AtUBC2 in yeast partially complemented the UV sensitivity conferred by deletion of *RAD6* (Zwirn et al., 1997). Since no evidence suggests that AtUbc2 interacts with yeast Rad18 or PCNA ubiquitination, the restoration of UV resistance could not be attributed to *AtUBC2* functioning in damage tolerance in yeast. Inactivation of single *AtUBC1*, *AtUBC2*, or *AtUBC3* has no obvious defect. Interestingly, only *ubc1 ubc2* double mutant, but not double mutants with *ubc3* showed early flowering via its defect in histone monoubiquitination (Cao et al., 2008; Gu et al., 2009). This observation suggests that *AtUBC1* and *AtUBC2* are functionally redundant, but *AtUBC3* plays a different role. Moreover, triple mutants of *ubc1,2,3* shows very severe developmental phenotypes. We still do not know whether AtUbc1, 2, 3 have a similar function in DDT to their counterpart Rad6 in budding yeast. Hence, it will be important to determine whether these mutants are sensitive to DNA damage.

Based on sequence analysis, Arabidopsis seems to lack a Rad18 homologue (Kunz, 2007). It is possible that there is a functional homologue of Rad18 in Arabidopsis rather than a sequence homologue. Another possibility is that Arabidopsis Rad6 homologue operates with a multi-protein E3 complex rather than a single Rad18-like E3 ligase. Indeed, there are a huge number of proteins forming a Skp1-Cul1-F-box complex of E3 ligase (Hotton and Callis, 2008). Thus, in plant a multi-protein ubiquitin ligase may substitute for Rad18 in damage tolerance.

AtUBC13A and *AtUBC13B* were identified encoding yeast Ubc13 counterparts. Physical interaction between AtUbc13 and the yeast or human Mms2 protein was shown (Wen et al., 2006). And also, the *AtUBC13* genes were able to complement the yeast *ubc13* null mutant for spontaneous mutagenesis and sensitivity to DNA-damaging agents. This suggests two Ubc13 counterparts in Arabidopsis may function similarly in budding yeast. Since the *Atubc13a Atubc13b* double mutant has spontaneous developmental phenotypes and is sensitive to

environments, it is difficult to investigate their response to DNA-damaging agents (R Wen, H Wang, W Xiao, unpublished data). On the other hand, this observation suggests that in Arabidopsis AtUbc13 has multiple functions other than its role in response to DNA damage. AtUbc13 appears to be involved in Fe signaling. *Atubc13a* mutant plants showed a perturbed expression of Fe-regulated genes and had deficiency in branched root hair formation, which is related to Fe deficiency (Li and Schmidt, 2010).

Four homologues of *UEV1* in Arabidopsis have also been isolated and named *AtUEV1A, B, C* and *D* (Wen et al., 2008). The critical residues in human or yeast Ubc13 or Mms2 are conserved in AtUbc13A/ B and AtUev1A-D, respectively. These specific residues have been shown critical for the Ubc13-Uev interaction, binding of ubiquitin to the Ubc13-Mms2 complex and the assembling of polyubiquitin chains by the Ubc13-Mms2 complex *in vitro* or cellular UV resistance (Eddins et al., 2006; Pastushok et al., 2005; Tsui et al., 2005). Indeed, each of AtUbc13A and AtUbc13B interacts with each of AtUev1A-D, and can also interact with yeast and human Mms2 or Uev1. With AtUbc13 or with Ubc13 from yeast or human, all four AtUev1 proteins can promote Ubc13-mediated Lys63 polyubiquitination (Wen et al., 2008). Furthermore, inactivation of *AtUEV1D* reduces seed germination and seedling growth in the presence of a DNA damaging agent MMS, and expression of *AtUEV1A-D* in yeast can fully complement yeast *mms2* deletion mutant (Wen et al., 2008). Collectively, these data suggests that AtUbc13 and AtUev1 form AtUbc13-AtUev1 polyubiquitinating complexes that participates in DNA damage tolerance. The four *AtUEV1A-1D* likely result from two sequential duplications via sequence analysis, and these two subgroups may be involved in different cellular processes, which needs to be investigated.

The cognate E3 of Ubc13, Rad5, is present in Arabidopsis. According to the result of

searching database via blasting the yeast Rad5 sequence, sequences related to yeast Rad5 have been identified in Arabidopsis and these genes were grouped previously into a putative *RAD5/RAD16*-like gene family (Shaked et al., 2006). Among them, AtRad5a and AtRad5b have the most significant identity and similarity (Chen et al., 2008). Both of them contain the conserved RING finger domain, characteristic of ubiquitin ligases and SWI/SNF helicase domains. Inactivation of *AtRAD5a* results in an enhanced sensitivity to cross-linking agents and MMS (Chen et al., 2008). Surprisingly, *Atrad5b* mutant plants did not show such sensitivity and the *Atrad5a Atrad5b* double mutant plants act like the *Atrad5a* mutant alone. Moreover, AtRad5a is involved in homologous recombination while *Atrad5b* is not (Chen et al., 2008). More work is needed to determine whether AtRad5b is still a functional homologue of yeast Rad5.

1.4 Objective of This Research

As discussed above, *uev1d* (Wen et al., 2008) and *rad5a* (Chen et al., 2008) single mutants are sensitive to DNA damaging agents. Since genes involved in yeast DDT are conserved in *Arabidopsis* (Kunz and Xiao, 2007) and mammals (Pastushok and Xiao, 2004), it suggests that the two-branch model of DDT is also conserved; however, how DDT operates in plants is unknown prior to this study. In budding yeast, mutations in TLS and error-free PRR have a synergistic effect, since *mms2/ubc13* or *rev1/rev3/rev7* single mutants are only moderately sensitive to DNA damaging agents, but a double mutant defective in both branches becomes extremely sensitive (Broomfield et al., 1998; Brusky et al., 2000; Xiao et al., 1999). Therefore, my research project aims to investigate whether this two-branch model of DDT exists in Arabidopsis. The specific objectives are:

(1) To create a double mutant defective in both branches of DDT.

(2) To test the sensitivity of the mutants in response to different types of DNA damages.

(3) To investigate the biochemical function of genes involved in DDT.

2. MATERIALS AND METHODS

2.1 Plasmid Construction

To confirm that the phenotype of *rad5a-3* attributes to inactivation of *AtRAD5A*, full length of *AtRAD5a* CDS was cloned into modified pBI121 (Clontech, Mountain View, CA, USA), a plant expression vector, and then transformed into *rad5a-3* mutants. First, *AtRAD5A* was amplified by polymerase chain reaction (PCR) using PfuUltra High-Fidelity DNA Polymerase (Stratagene, Cedar Creek, TX 78612, USA), sequence-specific oligonucleotide primer pairs (Table 2.1), *AtRAD5a* cDNA as a template. The thermal cycle conditions had 28 cycles. Each cycle consisted of 94℃ for 1 minute, 55 ℃ for 1 minute, and 72 ℃ for 3 minutes. PCR products were purified using Biobasic PCR Purification kit (Biobasic Molecular, East Markham, Ontario, Canada). The purified PCR products were digested with *Sal*I and *Not*I. The *Sal*I-*Not*I fragment was subjected to agarose gel electrophoresis and purification using the BioBasic Gel Purification Kit. Then the purified *Sal*I-*Not*I fragment was ligated into pre-digested modified pBI121 to yield the pBI121-AtRAD5a construct.

To analyze protein interaction with AtRad5a and AtUbc13a, two methods were applied: the yeast two-hybrid assay and protein pull-down assay. For preparing construct used in yeast two-hybrid assay, the same *Sal*I-*Not*I fragment of *AtRAD5a* was ligated into a modified pGAD424 (restriction sites modified by Dr. Xianzong Shi), a yeast two-hybrid vector, to make the pGAD424-AtRAD5a construct. pGBT9-AtUbc13a was kindly provided by Dr. Rui Wen. For protein expression in *E.coli*, a fragment containing the RING finger domain sequence of *AtRAD5a* and full-length *AtRAD5a* were ligated into expression vector pET28c (Invitrogen, Burlington, ON, CANADA), respectively.

All clones used in this study were confirmed by PCR screening and DNA sequencing.

Table 2.1 List of oligonucleotide primers

Primer Name	Primer Sequence
SW1	GTC AGT CGA CAA TGG GAA CGA AAG TCT CAG
SW2	ACT GGC GGC CGC TCA GGT AAA TAA CAT CTT GAG TTC C
SW31	CAC TTC CCT AGC ACA CTT C
SW32	TAC AGA ACT GCT GAT CAC
SW33	TGG ACA CTC TCC TTG CTC
HW503	TTC TTG TTC TAC CCC CTG C
HW504	GGA CAT AAC CCA GAA GTA G
HW507	CAT TCT CAT GAA ATT CAT GCG
HW508	GAC AGC TCT TGG GAA ACA C
HW539	CTC GGATCCAA GGA AAG TCT TCT GGC TTA G
HW540	CAGT GTCGAC TCA ATC AAG ACG GAC AAA TGA AAA G
HW669	CAGTCTCGACAATGGGTGCTTTCTGTTGTTGC
HW670	CAGTGCGGCCGCCTAAGAAGTTTCATTTTCATCAAA
LB1	GCG TGG ACC GCT TGC TGC AAC T

2.2 Plant Materials and Growth Conditions

A. thaliana ecotype “Columbia” and its mutant derivatives were used in this study. The rad5a and rev3 T-DNA insertion single mutant lines (SALK_124891, SALK_047150, SALK_049292 and SALK_029237) were obtained from the Arabidopsis Biological Resources Center. To generate the *rad5a rev3* double mutant, homozygous single mutant plants were obtained and crossed, and homozygous double mutants were identified in the F2 population by genomic PCR analysis. The plants were grown in pots placed in a growth room (21℃ constant,

16/8 h day/night photoperiod with a day light fluence rate of 140 μm/m^2/min). Genomic DNA was isolated and PCR performed as described below.

2.3 Plant Transformation

Agrobacterium tumefaciens containing desired pBI121-based constructs were used to transform *A. thaliana* as described (Wang et al., 2000). Arabidopsis plants were grown in the growth chamber with a density of 9 plants per pot. The growth conditions were 20℃ constant temperature and a photoperiod of 16/8 h (day/night). *A. tumefaciens* cells containing the plasmid of interest were grown on the 2×YT agar plate (1.6% peptone, 0.5% yeast extract, 0.5% NaCl, pH 7.0, 1.5% Agar) for three days. Then, the cells were harvested and resuspended in 300 ml of 1/2-strength Murashige and Skoog (MS) salts plus 5% sucrose. Before the infiltration, surfactant silwet-77 was added into the suspension to a final concentration of 0.01%. The inflorescence of 5-6 week-old plants were submerged into the suspension and then infiltrated under a vacuum of 600-700 mm Hg for 2 minutes. Infiltrated plants were put back into the growth chamber for setting seeds (T1). T1 seeds were screened on the plates of 1/2-strength MS salts medium (2.15 g/L MS salts (Sigma-Aldrich, Oakville, Ontario, Canada), 1% sucrose, 0.7% agar and pH 5.7) containing 50 μg/ml kanamycin and 300μg/ml Timentin. Kanamycin-resistant seedlings with exogenous plasmid were selected for further characterization.

2.4 Genomic DNA Isolation

Genomic DNA was prepared with a modified method as described (Edwards et al., 1991). A disc of *A. thaliana* leave was pinched out by the lid of a sterile eppendorf tube. Then 400 μl of extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added to the sample and gridded with a disposable plastic grinder until the entire tissue was ground. The tubes were centrifuged at 13,000 rpm for 5 minutes at 4 ℃ and 300 μl of

supernatant was transferred to a fresh 1.5 ml microcentrifuge tube. This supernatant was mixed with 300 µl isopropanol and left at room temperature for 10 minutes, which was followed by centrifugation at 13,000 rpm for 5 minutes. The supernatant was discarded and the pellet dried for about 10 min at room temperature. The pellet was then dissolved in 100 µl TE buffer or water. 2 µl of the sample were used for a 20 µl PCR reaction.

2.5 Polymerase Chain Reaction (PCR)

PCR was used to amplify DNA fragments for the purposes of cloning and other analyses. PCR reaction mixtures were created using the recipe guidelines in the instruction manual for the Taq DNA polymerase (Invitrogen). A PTC-100 programmable thermal controller (MJ Research, Inc., Watertown, MA) was used as the thermocycler to carry out the various amplifications. As a program guideline, a denaturing temperature of 94℃ for one minute was followed by an annealing temperature for 1minute, and primer extension was carried out at 72℃ for 1 minute per kilobase of DNA to be amplified. These three steps were repeated usually for a total of 30 cycles. The annealing temperature, extension time, total cycles may vary in different situations.

2.6 Total RNA Extraction and RT-PCR

Total RNA was isolated using TRIzol (Invitrogen) from 9-day-old Arabidopsis seedlings. Reverse transcript synthesis of the first-strand cDNA was performed with a ThermoScript RT-PCR kit (Invitrogen). Briefly, 2 to 4 µg of total RNA for each sample were reverse-transcribed with Moloney murine leukemia virus reverse transcriptase. Normally, the cDNA product of reverse-transcription was diluted 3 times, and 1 µl was used for a 20 µl PCR reaction.

2.7 Measurement of Plant Sensitivity to DNA Damaging Agents

Sterilized seeds were placed in 1/2 MS agar plates as previously described (Wen et al., 2008). For the seed germination assay, the medium was supplemented with different

concentrations of DNA-damaging agents and the plates were incubated in a growth chamber (continuous lighting at about 100 μm/m^2/min) for the period as indicated before photography.

For the root growth assay, the plates were incubated vertically in the growth chamber. After 9 days, the plates were photographed. The root length of each seedling was measured using NIH ImageJ Software (version 1.42) and expressed as the percentage of the average length of untreated wild-type roots in the same experiments.

A modified root-bending assay (Britt et al., 1993) was performed to assess plant sensitivity to UV light. Sterilized seeds were placed on the 1/2 MS agar plates and grown vertically for three days. The plates were then exposed to 1 kJ/m^2 of 254 nm UV light (FB-UVXL-1000 UV cross-linker, Fisher Scientific) every second day four hours after beginning of the light period for a total of three (for the 9th-day measurement) or four (for the 12th-day measurement) exposures. Immediately after the first exposure, the plates were rotated 90^o so that *de novo* root growth was redirected. The root growth after rotation was measured as described above.

2.8 Flow Cytometric Analysis

The seedlings of mutant and wild-type lines were grown vertically on 1/2 MS plates with or without MMS. Roots were removed from 9-day seedlings and the shoot part (almost entirely leaves) was used for flow cytometric analysis as described (Zhou et al., 2002). Briefly, fresh leaf tissue from mature Arabidopsis leaves was sampled in a 60-mm petri plate containing 400 μl of solution A of the High Resolution DNA kit-Type P (Partech, Munster, Germany). Tissue was chopped using a razor blade, and 1 ml of solution B containing DAPI was added for staining the nuclei. The suspension was then filtered through a 30-μm mesh. The sample was left for at least 5 min before being analysed using a Partec Ploidy Analyser (Partech). For each type of plant,

five to eight seedlings were measured individually. The average peak size (area) for various peaks of DNA contents (2C, 4C, 8C, etc) was obtained.

2.9 Yeast Strains and Cell Culture

S. cerevisiae wild-type strain DBY747 (*MAT**a**, his3*Δ*1, leu2-3,112 ura3-52 trp1-289*) and its isogenic mutant strains WXY382 (*rev3*Δ*::LEU2*), WXY731 (*rad5*Δ*::hisG-URA3-hisG*) and WXY736 (*rev3*Δ*::LEU2 rad5*Δ*::hisG-URA3-hisG*) were previously reported (Xiao et al., 2000). Yeast cells were grown in either the liquid YPD (1% yeast extract, 2% peptone, 2% dextrose) medium or on YPD+2% agar plates at 30^{o}C.

2.10 Testing Sensitivity of Yeast Cells to DNA-damaging Agents

A serial dilution assay as previously described (Barbour et al., 2006) was employed to determine yeast mutant sensitivity to MMS, 4NQO and MMC. For cisplatin-induced killing, overnight yeast cultures were used to inoculate fresh YPD. Cisplatin was added to the liquid culture at the given concentrations and samples were withdrawn at the indicated time. A serial dilution was made and spotted onto an YPD agar plate without the testing chemical. For UV sensitivity, serially diluted yeast samples were spotted on YPD, exposed to 254 nm UV light in the UV crosslinker at given doses and incubated in the dark.

2.11 Yeast Transformation

Yeast cells were transformed using a dimethyl sulfoxide (DMSO)-enhanced method as described (Hill et al., 1991) with some modifications. A 2 ml culture of yeast cells was grown overnight at 30 ℃ in a rich YPD medium, and sub-cultured into 3 ml of fresh YPD. When the yeast cells reached a mid-logarithmic phase of growth, they were harvested by centrifugation, washed in 400 μl LiOAc solution (0.1 M lithium acetate, 10 mMTris-HCl (pH 8.0), 1 mM EDTA), and resuspended in 100μl of the same solution. 5 μl denatured carrier DNA (single-

stranded salmon sperm DNA by incubating in boiling water for 5 minutes) and 0.1μg transforming DNA were added and mixed well. After incubation at room temperature for 5 minutes, 280 μl of 50% PEG4000 (50% polyethylene glycol 4000 in LiOAc solution) was added and mixed by inverting 4-6 times. Transformation mixture was then incubated for 30 minutes at 30 ℃. 40 μl of DMSO was then added, followed by a 5-minute heat shock in a 42 ℃ water bath. Yeast cells were then washed with sterile ddH_2O and resuspended in 100 μl of ddH_2O. The resuspended cells were plated on an appropriate minimal medium. The plates were incubated at 30 ℃ for 3 days for the colonies to grow.

2.12 Functional Complementation Assay

Gradient plate assays were performed for the semi-quantitative measurement of yeast cell sensitivity to MMS. At least three independent colonies from different transformants for each strain were individually inoculated into 1 ml of SD minimal medium. Following an overnight incubation, cell density was determined and equal numbers of cells from the transformants as well as controls were imprinted onto YPD alone or YPD gradient plates containing certain concentrations of MMS. An MMS gradient was formed by pouring 30 ml of YPD + MMS agar medium in a tilted square petri dish. The petri dish was placed flat after solidification followed by pouring a top layer of 30 ml YPD agar medium. 100 μl of overnight culture, mixed with 400 μl sterile water and 500μl of 2% molten YPD agar medium was printed onto the plates using a sterile microscope slide. Plates were incubated at 30 ℃ for overnight before being photographed.

2.13 Yeast Two-hybrid Analysis

The yeast two-hybrid strain PJ69-4A (James et al., 1996), received from Dr. P. James (University of Wisconsin, Madison, USA), was co-transformed with different combinations of Gal4 DNA-binding domain ($Gal4_{BD}$) and transcription-activating domain ($Gal4_{AD}$) constructs.

The co-transformed cells were initially selected on SD-Leu-Trp plates. For each transformation, at least three independent colonies were grown in SD-Leu-Trp plates and then replica plated onto either SD-Leu-Trp-His alone or SD-Leu-Trp-His with various concentrations of 1,2,4-amino triazole (3-AT) to test the activation of the P_{GAL1}-*HIS3* reporter gene, or SD-Leu-Trp-Ade to test the activation of the P_{GAL1}-*ADE2* reporter gene. Plates were incubated for at least two days at 30 ℃.

2.14 Protein Expression in *E. coli* and Protein Purification

pET28c-AtRAD5a and pET28c-AtRAD5a-RING were transformed into BL21(DE3)-RIL cells and grown overnight at 37 ℃ in the LB (Lennox Broth) plus ampicillin (Amp) medium and then sub-cultured 1:50 into LB + Amp the following day. Cells were allowed to grow to an optical density (OD) at 600 nm of between 0.6 and 0.8, induced with certain concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG) for overnight. The induced cells were harvested by centrifugation at 10,000 rpm in an Avanti Beckman JA10.5 rotor.

For protein purification, the harvested cells were resuspended in phosphate-buffered saline (PBS, 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3). Crude extracts were generated via passing the cells through a French Press at 10,000 psi. The soluble fraction was retained after centrifugation at 17,000 rpm in an Avanti Beckman JA17 rotor for 30 min. The soluble fraction was then run through a gravity-flow column for purification.

To a 1.5 ml microcentrifuge tube, 200 µl slurry of His•Bind Resin (Novagen, His•Bind purification kit) were added. The supernatant was removed after 1 minute of centrifuge at 1000 ×g. The resin was then washed to charge and equilibrated by washing or incubating with the following: 2 times with 2 volumes of sterile deionized water, 3 times with 2 volumes of 1 X charger buffer (8X = 400 mM NiSO4), and 2 times with 2 volumes of 1 X binding buffer (8X =

4 M NaCl, 160 mM Tris-HCl, 40 mM imidazole, pH 7.9). After these washes, pre-treated resin was incubated with soluble fraction from cell extracts at 4℃ for 1 hour. Then, the resin and soluble fraction were added into a gravity-flow column. When the liquid level reached the bed of resin, different buffers were added to wash and elute protein by follow steps: 3 times with 3 volumes of 1 X binding buffer, 2 times with 3 volumes of 1 X wash buffer (8X = 4 M NaCl, 480 mM imidazole, 160 mMTris-HCl, pH 7.9), 2 times with 3 volumes of 1 X elute buffer (4X = 4 M imidazole, 2 M NaCl, 80 mM Tris-HCl, pH 7.9). All the steps were carried out at 4℃. Purified proteins were kept at 4℃ for short-term use, or frozen quickly and kept at -70℃ for long-term storage.

2.15 GST Pull-down Assay

GST pull-downs were performed using MicroSpin GST Purification Modules (GE Healthcare, #27-4570-03). The purpose of this assay is to test the interaction between full length of AtRad5 or RING-finger domain of AtRad5 and AtUbc13A. Fifty microliters of purified GST and GST fusion proteins in 1X PBS were loaded and incubated in the purification module for 1 h at 4°C with gentle rocking. The module was then washed three times with 500 μl PBS. Subsequently, 50 μg of purified GST-AtUbc13 kindly given by Dr. Rui Wen in 1X PBS was added to the module separately and the incubation was continued for another hour at 4℃. The module was washed three times with 500 μl PBS, before 80 μl of reduced glutathione elution buffer were added to elute the affinity-purified proteins. Eluted samples were subjected to SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel) electrophoresis and visualized by Coomassie Blue staining.

2.16 Protein Electrophoresis and Western Blotting Analysis

Proteins were visualized using SDS-PAGE electrophoresis in the Mini-Protean 3 gel apparatus. In general, samples for SDS-PAGE were made by adding 2X protein sample buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 1.8% β-mercaptoethanol) and boiling 5 minutes. The samples were then cooled and loaded onto the protein gel. Usually 12% discontinuous (5% stacking, 12% separating) Tris-glycine polyacrylamide (37:1 acrylamide:bisacrylamide) gels were used. Gels were stained with a Coomassie Blue staining solution (0.025% Coomassie Brilliant Blue R250, 40% methanol, 7% acetic acid) for at least 15 minutes, followed by incubation in a de-stain solution (40% methanol, 10% acetic acid) until appropriate protein band could be visualized.

Following SDS-PAGE, the resolving gel was equilibrated for 20 min in a transfer buffer along with equal-sized polyvinylidenedifluoride (PVDF) membrane and 3M filter papers. The components were assembled as described in the manual for the Bio-Rad trans-blot semi-dry transfer cell, which was used for the transfer of proteins onto the PVDF membrane. Transfers were performed at a constant current of 1 mA/cm^2 for 1 hr. The membrane was then incubated in a blocking solution (5% skim milk) for 1 hour at 4℃. The primary monoclonal antibody against the His-tag antibody (Santa Cruz Biotechnology, Santa Cruz, CA 95060, USA) was diluted at 1:4000 in 10 ml PBS with 0.1% Tween 20 (PBST) containing 3% skim milk and incubated with the membrane at room temperature for 1 hour with gentle rocking. The membrane was washed 3 times for 15 minutes each with PBST. The secondary antibody was diluted at 1:10000 in 10 ml PBST containing 3% skim milk and incubated with the membrane for 1 hour. The membrane was then washed 3 times for 15 minutes each with PBST followed by 2 rinses each with PBS to prepare for the detection. The Western Lightening Chemiluminescence

Reagent (Perkin-Elmer, #NEL101) was used as the substrate for the visualization of horseradish peroxidase-conjugated secondary antibody.

3. RESULTS

3.1 Characterization of *rad5a rev3* Double Mutants

In budding yeast, mutations in TLS and error-free PRR have a synergistic effect. It is thus important to determine whether this two-branch DDT pathway is also conserved in plants. The only convenient and critical assay that can be applied to plants to date is to check synergistic effects in the mutants in which both error-free DDT and TLS branches are blocked. In the TLS branch, the choice of an *Atrev3* mutation to represent this branch is obvious since the *rev3* mutant is more sensitive to DNA damaging agents than *rev1* and *rev7*, and the *rev3* mutation is epistatic to *rev1* and *rev7* (Takahashi et al., 2005). In the error-free DDT branch, we found that while *ubc13* single mutants showed no noticeable phenotypes, the homozygous *ubc13a ubc13b* double mutant displayed multiple growth and developmental phenotypes in the absence of treatment with DNA damaging agents (data not shown), suggesting that Ubc13 may be involved in cellular processes beyond DNA damage response. This observation is understandable, as mammalian Ubc13 interacts with a panel of E3 ligases and is involved in several biological processes in addition to DNA damage responses (Andersen et al., 2005). Since a Uev is absolutely required for Ubc13-mediated Lys63-linked polyubiquitination (Hofmann and Pickart, 1999; McKenna et al., 2001), we suspect that the four *AtUEV1* genes may also have distinct as well as overlapping functions, making them unsuitable for the proposed investigation. We turned our attention to plant homolog(s) of Rad5, the yeast cognate E3 ligase of Ubc13 required for error-free DDT (Ulrich and Jentsch, 2000; Xiao et al., 2000), since E3s often provide substrate specificity.

After the identification of two *RAD5* homologs from Arabidopsis (Kunz and Xiao, 2007), our initial objective was to characterize *rad5a* and *rad5b* single and the double mutants and then test the hypothesis that a two-branch DDT pathway exists in plants. While this investigation was

in progress, Chen *et al*. reported that only *AtRAD5a* (*At5g22750*), but not *AtRAD5b* (*At5g43530*), is required for the DNA damage response. More importantly, the *Atrad5a Atrad5b* double mutant behaves same as the *Atrad5a* single mutant (Chen et al., 2008), suggesting that *AtRAD5b* does not provide a backup function for *AtRAD5a*. This report prompted us to focus on the genetic interactions between TLS represented by *AtREV3* and the putative error-free bypass pathway represented by *AtRAD5a*. Our rationale was that if the two pathways corresponding to these two genes constitute two parallel branches of DDT in plants, one might observe a strong synergistic interaction between *Atrad5a* and *Atrev3* mutations.

The *AtRAD5a* and *AtREV3* T-DNA insertion lines SALK_049292 and SALK_029237 were obtained from the Arabidopsis Biological Resource Center (ABRC, www.arabidopsis.org), and the alleles were designated *rad5a-3* in this study and *rev3-2* in a previous report (Sakamoto et al., 2003), respectively. The *rad5a-3 rev3-2* homozygous double mutants were generated through crossing *rad5a-3* and *rev3-2* plants. Characterization of the same *rev3-2* allele has been previously reported (Sakamoto et al., 2003). On the other hand, although two other *RAD5A* T-DNA insertion lines SALK_124891 (*rad5a-1*) and SALK_047150 (*rad5a-2*) have been reported (Chen et al., 2008), the *rad5-3* line utilized in this study has not been previously characterized. Sequence analysis of *rad5a-3* revealed that the T-DNA was inserted in the 14th exon of *AtRAD5a* (Fig. 3.1A). This location is at the 5’ and close to the T-DNA insertion site of *rad5a-2*, but 3’ to that of *rad5-1* (Fig. 3.2A). Genomic DNA PCR and RT-PCR utilizing a series of *AtRAD5a* gene-specific primers revealed that the genomic structure upstream and downstream of the T-DNA insertion at the *AtRAD5a* locus in the *rad5a-3* mutation remains intact (Fig. 3.1B, upper panel); however, the transcript level upstream of the T-DNA insertion was reduced while the downstream transcript was barely detectable (Fig. 3.1B). More importantly, both genomic

DNA PCR and RT-PCR failed to detect a wild-type band across the putative T-DNA insertion site (Fig. 3.1B). Given the fact that the T-DNA insertion in *rad5a-3* is at the 5' of the coding region for the RING-finger domain essential for the Rad5 ubiquitin ligase activity (Ulrich, 2003) and that *rad5a-3* mutant phenotypes are remarkably similar to that of *rad5a-1* and *rad5a-2* (Fig. 3.2C and D), we are content that *rad5a-2* is a null mutation.

To characterize the *rad5a-3 rev3-2* double mutant, gene-specific primers for *AtRAD5a* and *REV3* plus a primer specific to the left-border sequence of T-DNA were used to confirm the insertion of T-DNA (Fig. 3.1C). To further confirm that the expression of full-length *AtRAD5a* and *AtREV3* was abolished in the double mutant by the T-DNA insertion, total RNA was extracted from the seedlings and analyzed by RT-PCR. A DNA band representing the *AtRAD5a* transcript was detected from wild-type and *rev3-2* plants but not from *rad5a-3* or the double-mutant plants. Similarly, the *REV3* transcript was detected from wild-type and *rad5a-3* plants but not from *rev3-2* or the double-mutant plants (Fig. 3.1D).

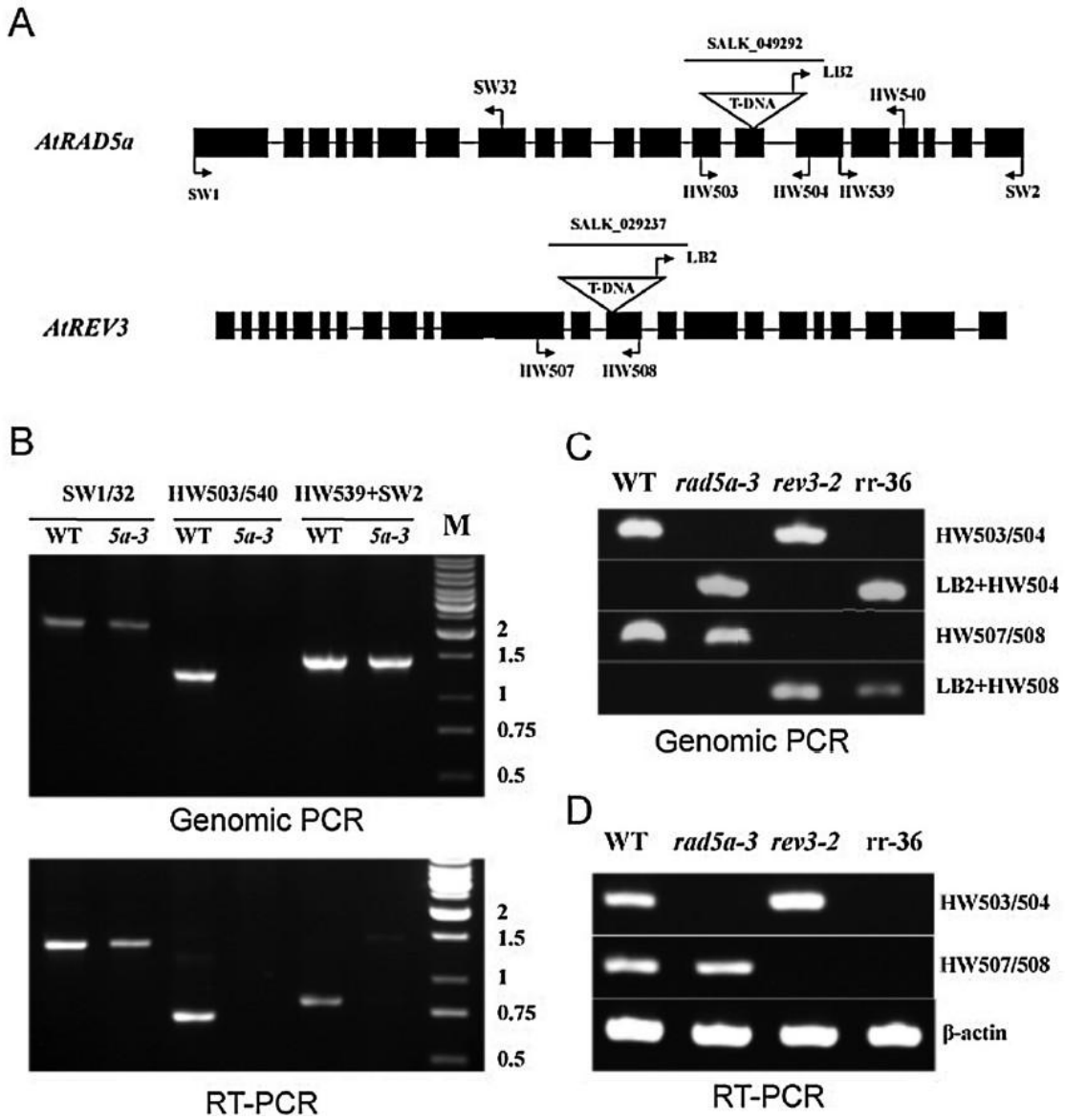
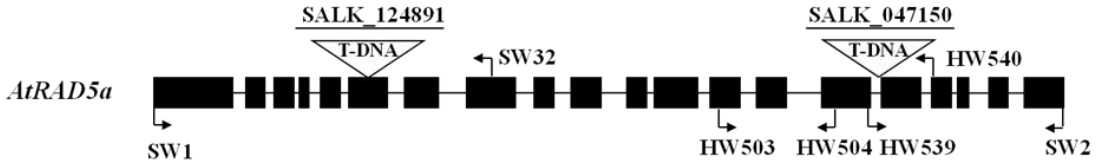
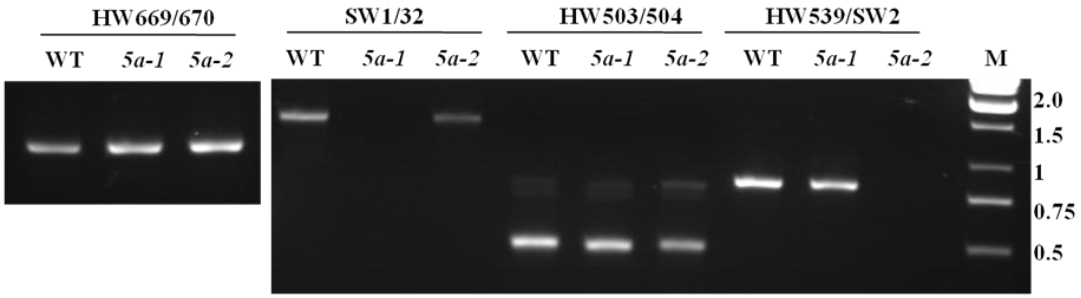

A
SALK_049292
LB2
SW32
T-DNA
HW540
AtRAD5a
SW1
HW503
HW504
HW539
SW2
SALK_029237
LB2
T-DNA
AtREV3
HW507
HW508
B
SW1/32
HW503/540
HW539+SW2
WT
5a-3
WT
5a-3
WT
5a-3
M
2
1.5
1
0.75
0.5
Genomic PCR
2
1.5
1
0.75
0.5
RT-PCR
C
WT
rad5a-3
rev3-2
rr-36
HW503/504
LB2+HW504
HW507/508
LB2+HW508
Genomic PCR
D
WT
rad5a-3
rev3-2
rr-36
HW503/504
HW507/508
β-actin
RT-PCR

Figure 3.1 Confirmation of *rad5a* and *rev3* T-DNA insertion mutants. (A) Genomic structure showing the location of primers and insertion sites of T-DNA in *rad5a-3* and *rev3-2*. Closed boxes: exons; lines, introns; SW1, SW2, SW32, HW503, HW504, HW539 and HW540, gene-specific primers for *AtRAD5a*; HW507 and HW508, gene-specific primers for *AtREV3*; LB2, T-DNA left border primer. (B) Characterization of *rad5a-3* T-DNA insertion mutation through genomic DNA PCR and RT-PCR. Primers and plant lines used in both reactions are indicated on the upper panel. Wild-type (WT) and *Atrad5a-3* (*5a-3*) samples were treated under identical experimental conditions. (C) Genomic DNA PCR to confirm the *rad5a-3* and *rev3-2* alleles in *rad5a-3* and*rev3-2* single mutants and the corresponding double mutant (rr-36). Primers used for genomic DNA PCR are indicated on the right panel. (D) RT-PCR analysis of*rad5a-3*and*rev3-2*single mutants and the *rad5a-3 rev3-2* double mutant line rr-36.Amplification of a β-actin cDNA was used as a reference. Primers used in each reaction are indicated on the right of the panel.

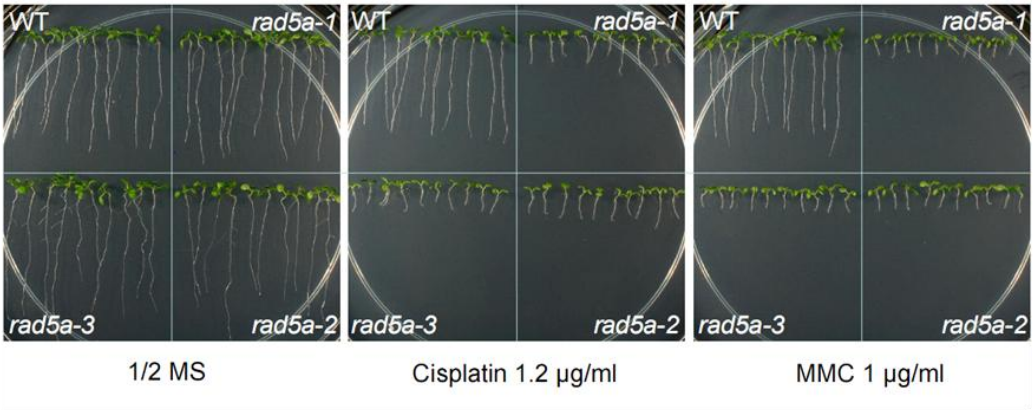
A


SALK_124891
T-DNA
SW32
SALK_047150
T-DNA
HW540
AtRAD5a
SW1
HW503
HW504
HW539
SW2

B


HW669/670
SW1/32
HW503/504
HW539/SW2
WT
5a-1
5a-2
M
2.0
1.5
1
0.75
0.5

C


WT
rad5a-1
rad5a-3
rad5a-2
1/2 MS
Cisplatin 1.2 μg/ml
MMC 1 μg/ml

D

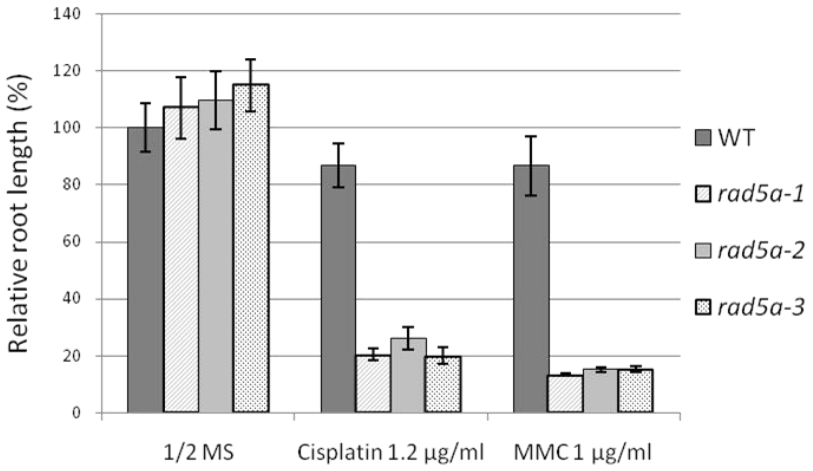
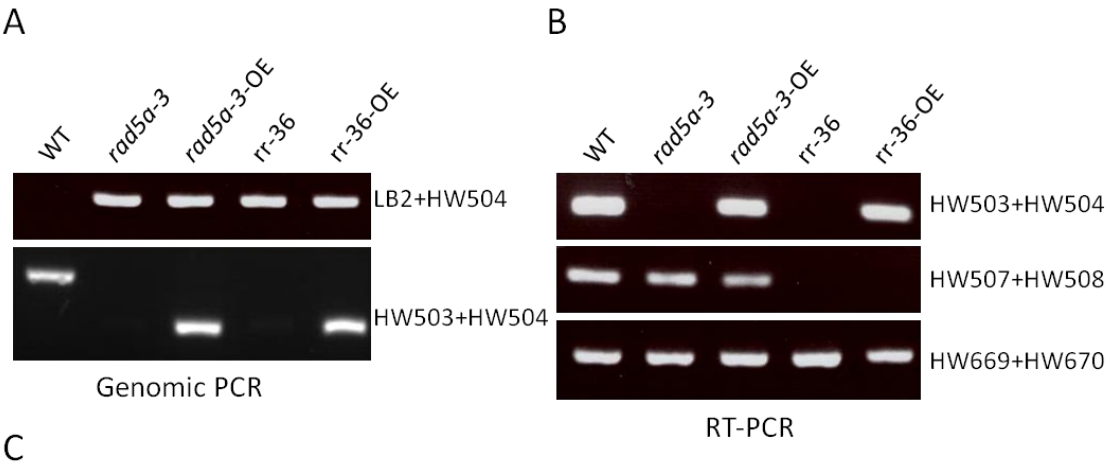

Relative root length (%)
140
120
100
80
60
40
20
0
1/2 MS
Cisplatin 1.2 μg/ml
MMC 1 μg/ml
WT
rad5a-1
rad5a-2
rad5a-3

Figure 3.2 Comparison of *rad5a-3* and other two alleles: *rad5a-1* and *rad5a-2***.** (A) Genomic structure showing the location of primers and insertion sites of T-DNA in *rad5a-1* and *rad5a-2*. Closed boxes, exons; lines, introns; SW1, SW2, SW32, HW503, HW504, HW539 and HW540, gene-specific primers for *AtRAD5a*. (B) Characterization of *rad5a-1* and*rad5a-2* T-DNA insertion mutation through RT-PCR. Primers and plant lines used in both reactions are indicated on the upper panel. Wild-type (WT) and *Atrad5a-1* (*5a-1*) and *Atrad5a-2* (*5a-2*) samples were treated under identical experimental conditions. HW669 and HW670, gene-specific primer for an unrelated gene *At5g38895* (C) Sensitivity of wild-type and three alleles of *rad5a* single mutants to DNA crosslinking agents cisplatin and MMC by root growth assay. Synchronized seeds were sown onto the 1/2 MS agar plates with or without the agents at the concentrations as indicated. (D) Quantitative analysis of root growth after treatment of cisplatin and MMC. Each bar represents an average of at least three replicates each containing 10 measurements. Error bars indicate standard deviations.

Additionally, to confirm that the phenotypes of *rad5a-3* were attributed to the inactivation of *AtRAD5a*, plasmid pBI121-AtRAD5a was transformed into *rad5a-3* single mutants as well as *rad5a-3 rev3-2* double mutants for complementation by overexpression (OE) of *AtRAD5a* driven by the 35S promoter. *rad5a-3*-OE or rr-36-OE refers to the *rad5a-3* single mutants or *rad5a-3 rev3-2* double mutants transformed with pBI121-AtRAD5a, respectively. The transformed *AtRAD5a* DNA fragment and its transcript were detected in *rad5a-3*-OE and rr-36-OE transgenic plants but not in the *rad5a-3* single or *rad5a-3 rev3-2* double mutants (Fig. 3.3A and B). These observations confirmed that *AtRAD5a* was transformed and expressed in the mutant plants. Furthermore, in the presence of 2 μM or 4 μM cisplatin, these transgenic plants displayed increased resistance to cisplatin than their corresponding mutant hosts in a root growth assay (Fig. 3.3C). In contrast, the seedlings of unrelated transgenic line S811 did not shown any resistance to the DNA-damage agents compared to the wild-type plants (Fig. 3.3C), indicating that the increased resistance of *rad5a-3*-OE and rr-36-OE transgenic plants is due to the overexpression of *AtRAD5a*. These results collectively confirm that *rad5a-3* mutants are defective in *AtRAD5a*-encoded function(s).


A
WT
rad5a-3
rad5a-3-OE
rr-36
rr-36-OE
LB2+HW504
HW503+HW504
Genomic PCR
B
WT
rad5a-3
rad5a-3-OE
rr-36
rr-36-OE
HW503+HW504
HW507+HW508
HW669+HW670
RT-PCR

C

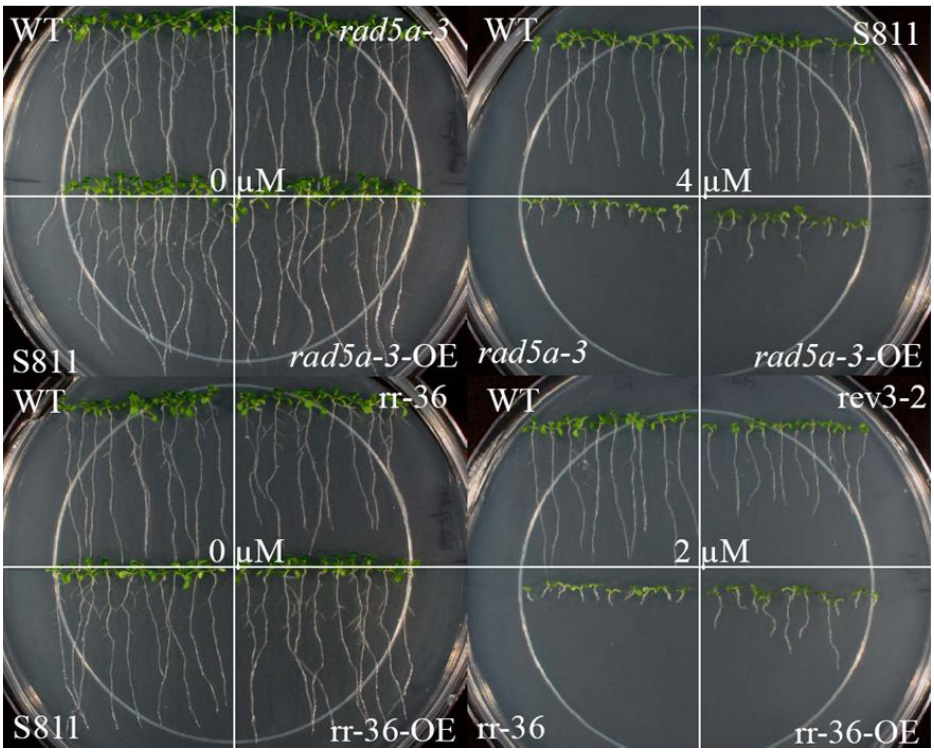

WT
rad5a-3
0 μM
S811
rad5a-3-OE
WT
rr-36
0 μM
S811
rr-36-OE
WT
S811
4 μM
rad5a-3
rad5a-3-OE
WT
rev3-2
2 μM
rr-36
rr-36-OE

Figure 3.3 Complementation of *rad5a-3* and *rad5a-3 rev3-2* mutants by *AtRAD5a* overexpression. (A) Characterization of *rad5a-3*-OE and *rr-36*-OEtransgenic plants through genomic DNA PCR. (B) Characterization of *rad5a-3*-OE and *rr-36*-OE transgenic plants through RT-PCR. Plant lines used in both reactions are indicated on the upper panel, and primers used are indicated on the right panel. All the samples were treated under identical experimental conditions. *rad5a-3*-OE and rr-36-OE refer to the *rad5a-3* single mutants and *rad5a-3 rev3-2* double mutants transformed with pBI121-AtRAD5a, respectively. (C) Sensitivity of wild-type, *rad5a-3* single, *rad5a-3 rev3-2* double mutants and their transformants to the DNA crosslinking agent cisplatin by root growth assay. Synchronized seeds were sown onto the 1/2 MS agar plates with or without the agents at the concentrations as indicated. S811 refers to an unrelated overexpression line in the wild-type background.

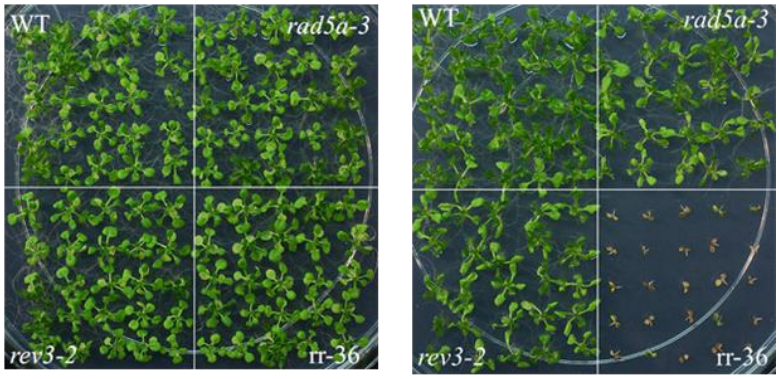
3.2 Genetic Interaction between *AtRAD5a* and *AtREV3* in Response to Different DNA-damaging Agents

3.2.1 Effects of *rad5a* and *rev3* in Response to MMS-induced killing

It has been reported that *rad5a* and *rev3* single mutants show no morphological changes under normal growth conditions compared to the wild-type plants; however, they display characteristic sensitivity to DNA-damaging agents (Chen et al., 2008; Sakamoto et al., 2003). We were able to confirm these reported phenotypes (See Figs. 3.4 and 3.6). Our objective of this study was to evaluate genetic interactions between *RAD5a* and *REV3* with respect to growth inhibition by selected DNA-damaging agents. We chose MMS as the primary agent since it specifically causes a replication-blocking lesion 3-methyladenine (3MeA) on DNA that largely relies on DDT for cell survival in a model yeast system (Broomfield et al., 1998). It is of great interest to note that *rev3* (Sakamoto et al., 2003) and *rad5a* (Chen et al., 2008) single mutants displayed very moderate levels of sensitivity to MMS. Using an MMS dose range that did not have obvious growth effects on the single mutants, we were able to show that the *rad5a-3 rev3-2* double mutant was particularly sensitive to growth inhibition by MMS. For example, in a plate containing 50 ppm MMS, seed germination of neither single mutant was affected, whereas all the double mutant seeds were unable to germinate (Fig. 3.4A). Similarly, in a root growth assay, MMS concentrations up to 20 ppm did not inhibit wild-type or single mutant growth; in sharp contrast, root growth of the double mutant was inhibited by more than 90% (Fig. 3.4B). Quantitative analysis shows that MMS doses that result in root lengths that are 30% of normal in wild-type, *rad5a*, *rev3* and the double mutant plants are 40, 36, 29 and 9 ppm, respectively (Fig. 3.4C), indicating that the genetic interaction between *rad5a* and *rev3* is greater-than-additive and that *AtRAD5a* and *AtREV3* likely function in alternative pathways to deal with the same lethal

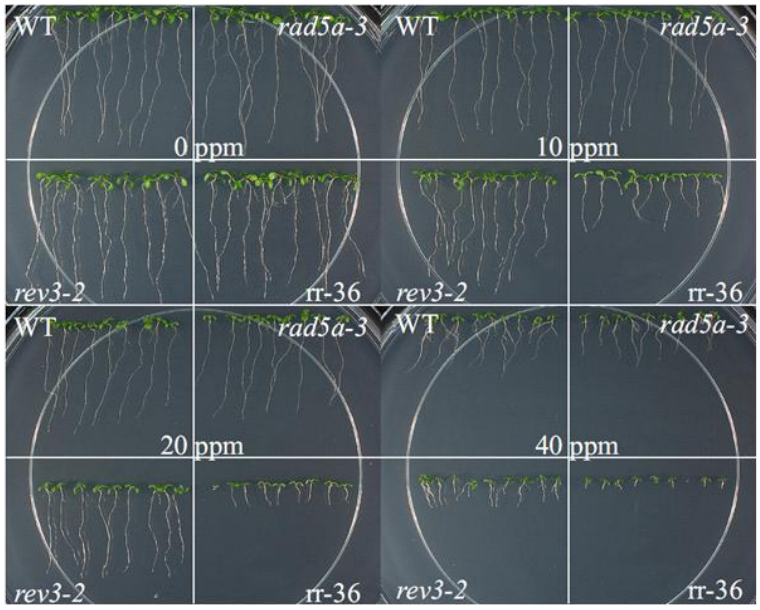
lesion(s) induced by MMS. The above observed phenomenon is unlikely due to variation among seed lots or a clonal effect, since two different double mutant lines derived from the original cross between the single mutants displayed similar phenotypes (Fig. 3.4C).

A



no MMS 50 ppm MMS

B



C

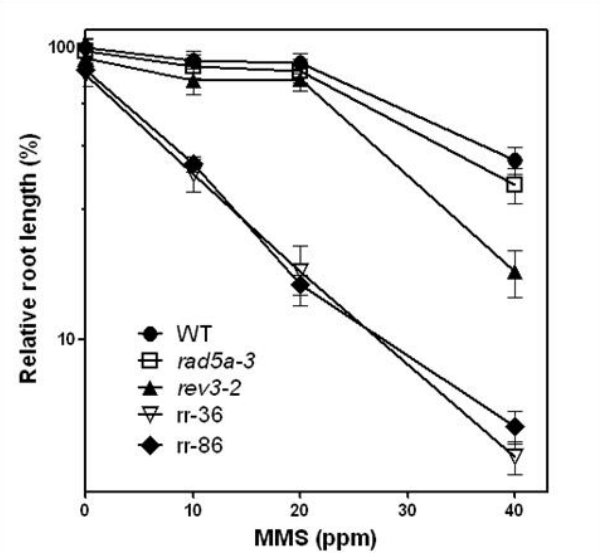


Figure 3.4 Sensitivity of wild-type, *rad5a*, *rev3* single and *rad5a rev3* double mutants (rr-36 and rr-86) to MMS. (A) Sensitivity of wild-type plants and mutants to MMS by a seed germination assay. Synchronized seeds were sown onto the 1/2 MS agar plates with or without MMS as indicated. Pictures were taken after a 20-day incubation. (B) Root growth of wild-type and mutants in the presence of different concentrations of MMS. Pictures were taken after 9-day incubation. (C) Quantitative analysis of root growth as shown in (B). Root length was expressed as the percentage of the average length of untreated wild-type roots. Each datum point represents an average of three replicates each containing 9 plants. Error bars indicate standard deviations.

3.2.2 Effects of *rad5a* and *rev3* in Response to UV- and 4NQO-induced Killing

The *rev3* mutant was described as highly sensitive to UV irradiation (Sakamoto et al., 2003), but whether *rad5a* is sensitive to UV is unclear prior to this study. Following the normal protocol of the root bending assay (Britt et al., 1993) with different doses of UV irradiation, I was unable to detect additional sensitivity in the double mutant. Wild-type and the *rad5a* single mutant grew similarly after UV irradiation, while *rev3* and the double-mutant plants displayed similar levels of sensitivity to UV (data not shown). The above observation suggests that *AtRAD5a* does not contribute to tolerance of UV-induced DNA damage under our experimental conditions. We envisage that in previous assays where *rad5a-3* displayed enhanced sensitivity to DNA damaging agents, plants were continuously exposed to the mutagens (Chen et al., 2008), whereas UV treatment in the above experiment was transient. To address whether chronic *vs*. acute treatment caused different responses of *rad5a* mutants to UV and MMS, we employed two strategies. First, we developed a protocol that allowed multiple exposures of plants to UV. As shown in Fig. 3A, although the *rad5a-3* single mutant still did not display sensitivity to UV, the *rad5a-3 rev3-2* double mutant showed moderate but noticeable additional sensitivity compared with the *rev3-2* single mutant (Fig. 3.5A, B). Our second strategy was to treat plants with a UV-mimetic agent 4NQO. Both single mutants displayed increased sensitivity to 4NQO over essentially the entire dose range examined, and the two mutations are apparently additive with respect to root growth in the presence of 4NQO (Fig. 3.5C, D). For example, at a dose of 0.1 μg/ml 4NQO treatment, the root growth of *rad5a-3* and *rev3-2* single mutants was inhibited by 38% and 73%, respectively. If the two mutation effects were additive, an expected growth inhibition compared to the wild-type plant would be 83% (（Wt 85%- *rad5a* 61%）+ （Wt 85%- *rev3* 26%）), which agrees very well with the observed 85% inhibition of the *rad5a-3*

rev3-2 double mutants (Fig. 3.5D). Hence, AtRev3 appears to play a major role in bypassing UV- and 4NQO-induced lesions, although AtRad5a also contributes to the bypass, particularly when AtRev3 is unavailable.

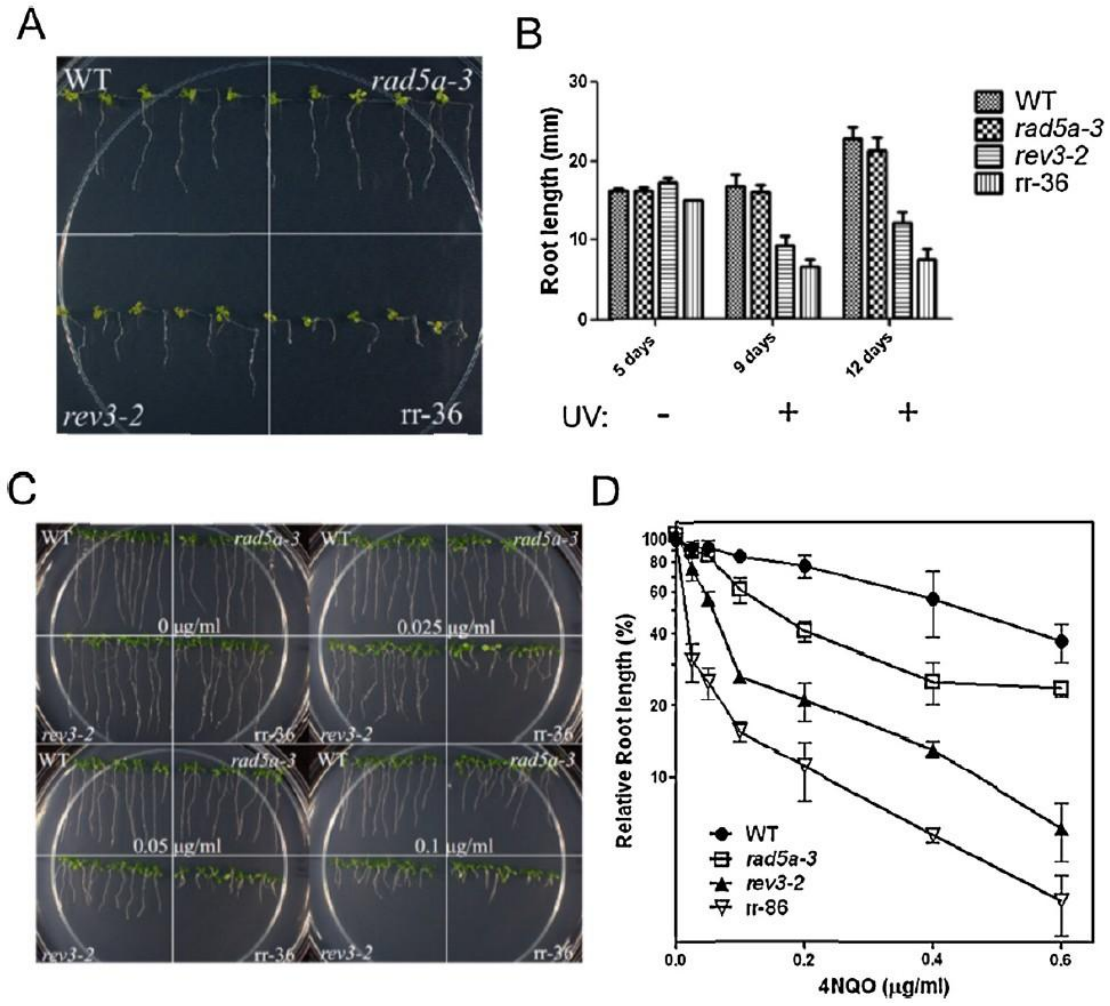


Figure 3.5 Sensitivity of wild-type, *rad5a*, *rev3* single and *rad5a rev3* double (rr-36) mutants to UV and 4NQO. (A) Representative root growth images after exposures to UV. After 3 days of germination, the seedlings were repeatedly exposed to1 kJ/m^2 UV irradiation in two-day intervals and incubated in a growth chamber. The picture was taken after four exposures and a 12-day incubation. (B) Quantitative analysis of root growth after repeated UV irradiations. Each datum point represents three replicates each containing five plants. Error bars indicate standard deviations. (C) Representative root growth images of wild-type and mutants without or with different concentrations of 4NQO as indicated. Pictures were taken after a 9-day incubation. (D) Quantitative analysis of root growth after 4NQO treatment. Each datum point represents an average of at least three replicates each containing 9 measurements. Error bars indicate standard deviations.

3.2.3 Effects of *rad5a* and *rev3* in Response to Cisplatin- and MMC-induced Killing

Both *rev3* (Sakamoto et al., 2003; Takahashi et al., 2005) and *rad5a* (Chen et al., 2008) mutants have been reported to confer enhanced sensitivity to crosslinking agents like cisplatin and MMC. When plants were treated with doses at lower levels than previously reported, we found that the effects of two mutations are also strongly additive with respect to killing by cisplatin and MMC. For example, in the presence of 0.6 μg/ml cisplatin (Fig. 3.6A) or 0.5 μg/ml MMC (Fig. 3.6B), seed germination of single mutants was apparently normal; however, the germination of *rad5a-3 rev3-2* double mutant seeds was severely inhibited.

Quantitative analysis indicates that the effects of *rad5a* and *rev3* mutations on root growth (Fig. 3.7) in response to low doses of cisplatin and MMC were greater than additive. For example, at a dose of 0.15 μg/ml cisplatin, the growth of neither single mutant was inhibited, whereas the double mutant root growth was inhibited by 90% (Fig. 3.7A, B). A Similar effect was also observed after 0.25 μg/ml MMC treatment (Fig. 3.7C, D). After treatment with increasing doses of crosslinking agents, each single mutant displayed enhanced sensitivity and the effects of *rad5a-3* and *rev3-2* appeared to be additive (Fig. 3.7). It was observed that root growth in the double mutant was inhibited by more than 90% at low to moderate concentrations of Cisplastin (0.3 μg/ml) and MMC (0.5 μg/ml). Since root growth was almost completely inhibited and the residue root growth might mostly come from cell elongation rather than cell proliferation, there would be little further increase in the inhibition response for the double mutant at higher concentrations of Cisplatin and MMC. Thus, a synergistic effect could only be observed at low concentrations. Nevertheless, these results suggest that both AtRad5a and AtRev3 play important roles in surviving lesions induced by DNA cross-linking agents, but their functions or substrate specificity may not completely overlap.

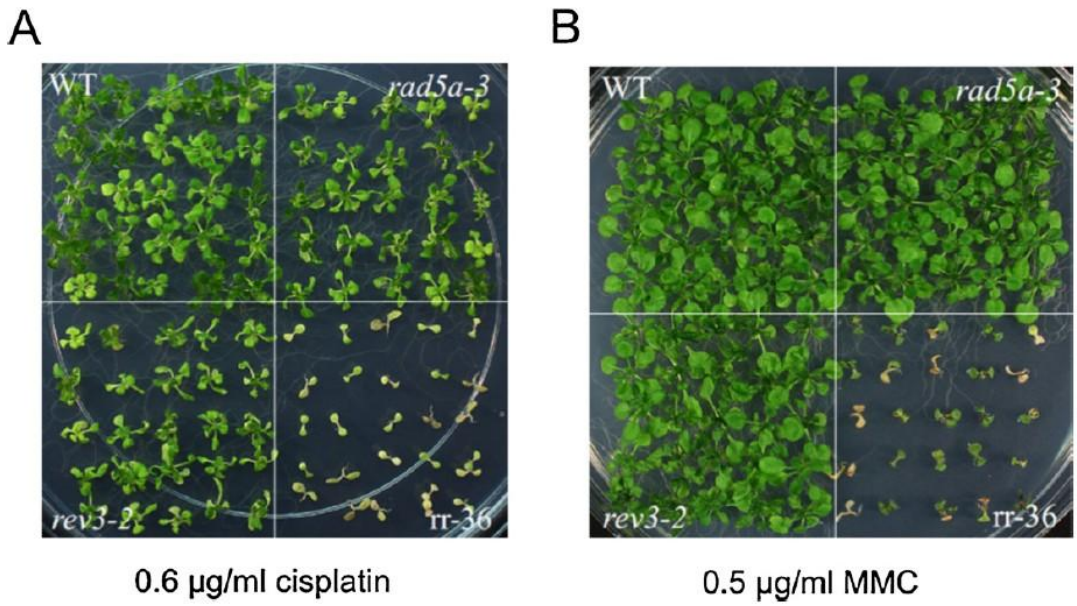


Figure 3.6 Sensitivity of wild-type, *rad5a*, *rev3* single and *rad5a rev3* double (rr-36) mutant to DNA crosslinking agents by a seed-germination assay. (A) cisplatin. (B) MMC. Synchronized seeds were sown onto the 1/2 MS agar plates with or without the agents at the concentrations as indicated. Pictures were taken after a 20-day incubation. All seedlings grew well on plates without DNA damaging agents as shown in Fig. 3.4A.

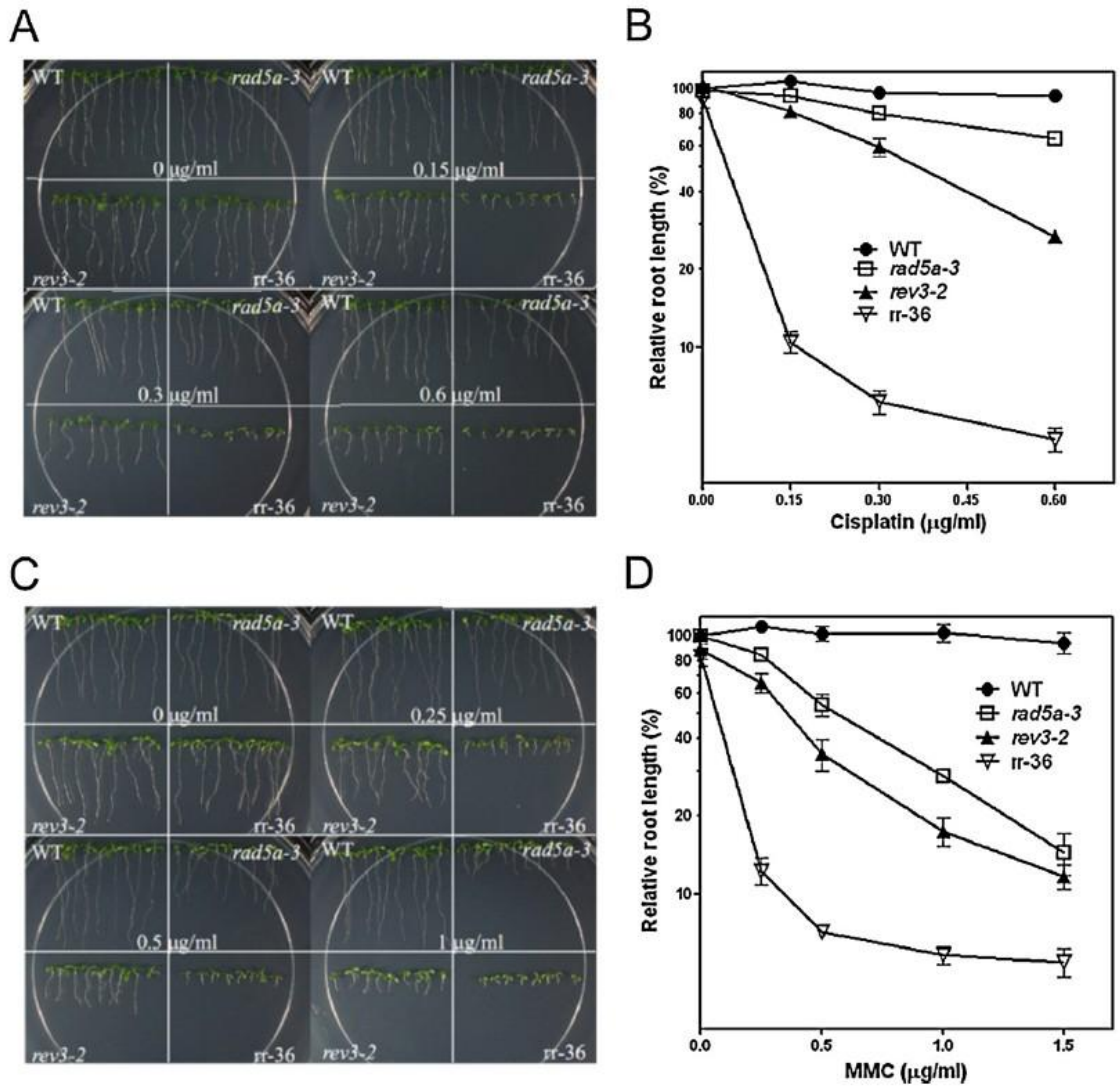


Figure 3.7 Sensitivity of wild-type and *rad5a*, *rev3* mutant seedlings to DNA crosslinking agents by a root growth assay. Representative root growth images of wild-type and mutants are shown with different concentrations of (A) cisplatin and (C) MMC. Pictures were taken after 9-day incubation. Quantitative analysis of root growth in the presence of (B) cisplatin and (D) MMC. Each datum point represents an average of three replicates each containing 9 plants. Error bars indicate standard deviations.

3.3 Relative Contributions of Plant and Yeast *RAD5* and *REV3 to* DNA Damage Response

Throughout this study, we consistently observed that *rev3-2* mutant plants were more sensitive to various DNA damaging agents than *rad5a-3* plants. This was surprising since our past experience suggests that in budding yeast, the *rad5* mutant is much more sensitive to various DNA damaging agents than *rev3*. To critically examine this issue and also to compare genetic interactions between *REV3* and *RAD5* in yeast and in plants, we made isogenic *rev3* and *rad5* single and double yeast null mutants and examined their relative sensitivity to all DNA damaging agents employed in this study. As seen in Fig. 3-8, all yeast single and double mutants grew equally well in the absence of treatment with DNA damaging agents. The *rad5* mutant is significantly more sensitive to MMS, UV and 4NQO than *rev3*, but the two mutations only showed synergistic interaction with MMS treatment, while they are additive to killing by UV and 4NQO. Interestingly, unlike *rev3*, the *rad5* single mutant did not display noticeable sensitivity to cisplatin or MMC, but the double mutant is more sensitive to these crosslinking agents than the *rev3* single mutant. Hence, yeast Rad5 is required for the tolerance to DNA crosslinks only when the Rev3 function is unavailable.

3.4 Simultaneous Inactivation of *AtRAD5a* and *AtREV3* Accumulates Polyploid Cells in the Presence of a Sublethal Dose of MMS

Endopolyploidy, the existence of polyploid cells in diploid organisms, is a common feature in plants and is believed to serve important biological functions in different cells and tissues (Barow, 2006). Endoreduplication is considered a variant form of the cell

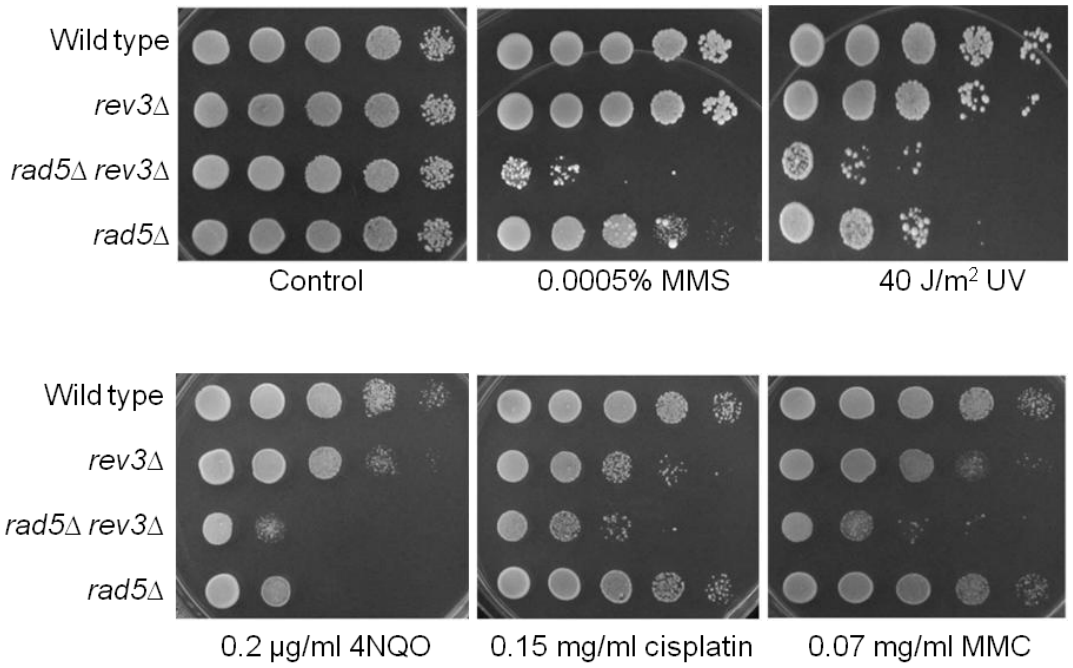


Figure 3.8 Sensitivity of wild-type and DDT-mutant yeast to different DNA-damaging agents by a 10-fold serial dilution assay. Overnight cultures were diluted and spotted on YPD or YPD plus different concentrations of DNA damaging agents as indicated. For UV irradiation, spotted plates were exposed to the UV dose as indicated. For cisplatin treatment, cells in a liquid culture were incubated with different concentrations of cisplatin for 50 min., washed, diluted and spotted on YPD. Pictures were taken after a 2-day incubation of the plates. For each DNA-damaging agent, treatment with multiple doses was performed but only one representative plate for each agent treatment is shown.

cycle in which mitosis is inhibited while DNA replication is permitted. Little is known about the effect of DNA damage on endoreduplication in plants. If *AtRAD5a* and *AtREV3* function as two branches within the DDT pathway to bypass MMS-induced replication blocking lesions, one would predict that in the absence of both *AtRAD5a* and *AtREV3* functions, mitosis could be inhibited in the presence of incompletely replicated genome, most likely resulting in cells reminiscent of endoreduplication. To test this hypothesis, 9-day old seedlings of wild-type and mutants plants with or without MMS treatment were analyzed for the nuclear DNA content by flow cytometry. Without treatment, the wild-type plants and the mutants showed similar profiles of DNA contents as previously reported (Galbraith et al., 1991). There were four major peaks at the 2C, 4C, 8C and 16C level and a minor peak at the 32C level (data not shown). Interestingly, the peaks shifted to 16C and 32C when the *rad5a rev3* double mutant plants were treated with 25 ppm MMS (Fig. 3.9A). Quantitative analysis was performed on the wild-type and mutant plants with or without MMS treatment. With 25 ppm MMS treatment, the percentage of nuclei with 16C and 32C DNA contents increased dramatically in the *rad5a rev3* double mutants, while the percentages of nuclei with 2C and 4C DNA contents decreased (Fig. 3.9B). In contrast, the wild-type and single mutants had no apparent shift in the flow cytometry profile (Fig. 3.9B). These data indicate that in the presence of a sub-lethal dose of MMS, either the *AtRAD5a*-mediated pathway or *AtREV3*-mediated pathway can bypass replication-blocking lesions; however, when both pathways are inactivated, cells suffer from incomplete replication and reduced rounds of cell division.

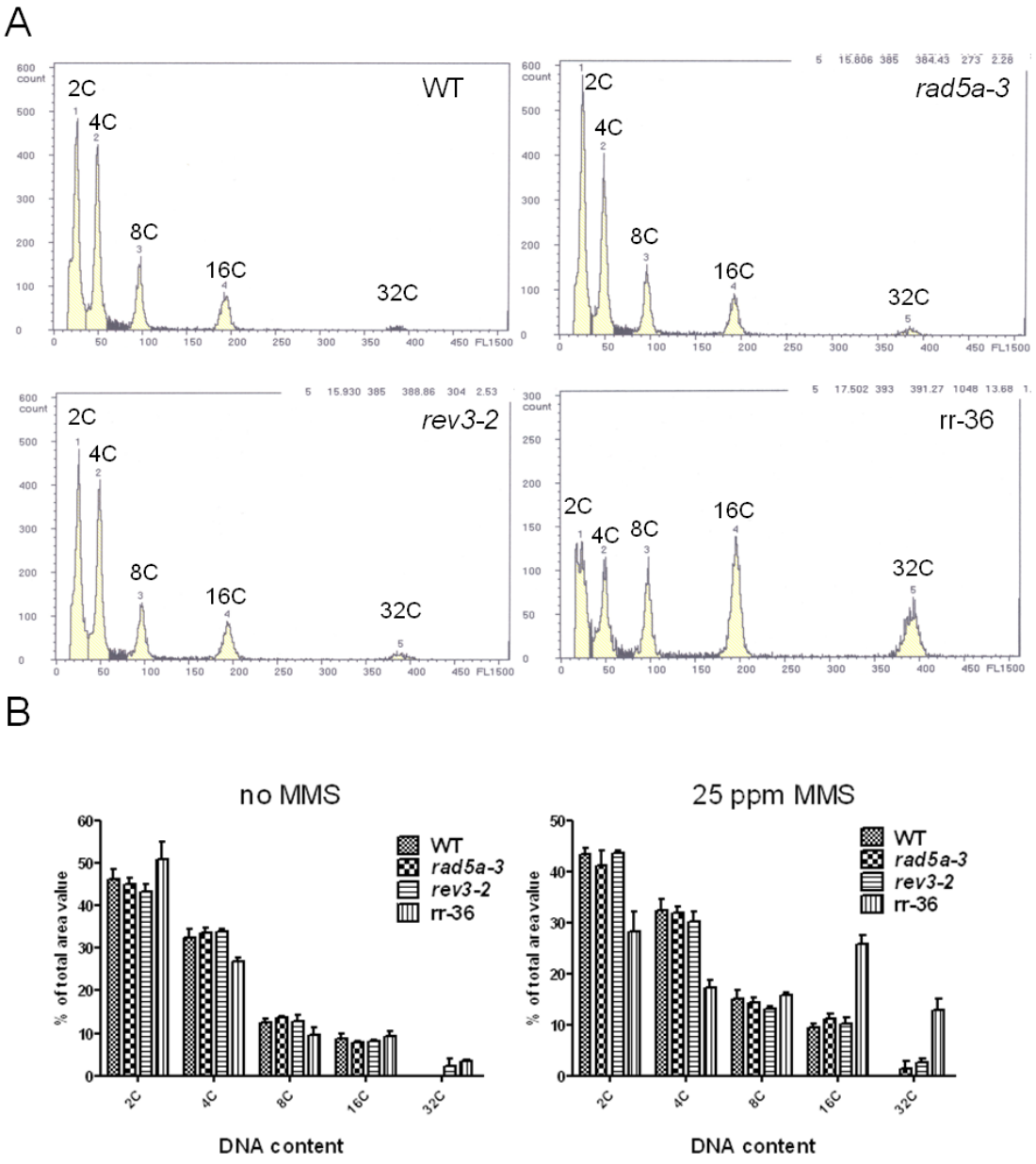


Figure 3.9 Flow cytometric analysis of wild-type, *rad5a*, *rev3* single and *rad5a rev3* double mutant (rr-36). (A) Representative flow cytometric data after 25 ppm MMS treatment. Five peaks indicate DNA contents of 2C, 4C, 8C, 16C and 32C. (B) Quantitative analysis of nuclear DNA content. Each determination for a particular DNA content peak represents the average of five to eight individual plants and is expressed in percentage, with the total value for all peaks adding up to 100%.

3.5 Physical Interaction between AtRad5a and AtUbc13

In budding yeast, Rad5a is the cognate E3 of Ubc13, and it interacts with Ubc13 through its RING-finger domain (Ulrich and Jentsch, 2000). In Arabidopsis, all the data collected suggests that AtRad5a is the structural and functional homologue of yeast Rad5. To investigate the protein-protein interaction between AtRad5a and AtUbc13a, two different methods were applied. The first one is yeast two-hybrid assay. The *AtUBC13A* ORF was cloned into a Gal4 DNA-binding domain vector pGBT9. The *AtRAD5a* ORF and a 450-bp fragment of *AtRAD5a* (nt. 2281-2730) encoding the RING-finger domain (amino acids 761-910) were cloned into a Gal4 transcription-activating domain vector pGAD424 (pGBT9-AtUbc13a and pGAD424-AtRad5aRING were kindly provided by Dr. Rui Wen). However, under the present experimental conditions, no interaction between either AtUbc13a and either AtRad5a or between AtUbc13a and RING-finger domain of AtRad5a could be detected (data not shown).

Another method is to detect their interaction through an *in vitro* pull-down assay using purified His-tag protein. The *AtRAD5a* ORF and the 450-bp fragment of *AtRAD5a* were also cloned into His-tag protein expression vector pET28c. However, only His-AtRad5aRING was induced by IPTG with an expected size around 22kDa. The concentration of IPTG had no effect on the yield of protein expression (Fig. 3.10). His-AtRad5aRING was detected in the soluble fraction (Fig. 3.10B). Subsequently, His-AtRad5aRING was expressed in a large-scale bacterial culture and purified. The purified His-AtRad5aRING was added to a column bound with either GST or GST-AtUbc13a which are kindly given by Dr. Rui Wen. After incubation, washing and elution, His-AtRad5aRING was not found to be co-eluted with GST or GST-AtUbc13a (Fig. 3.11).

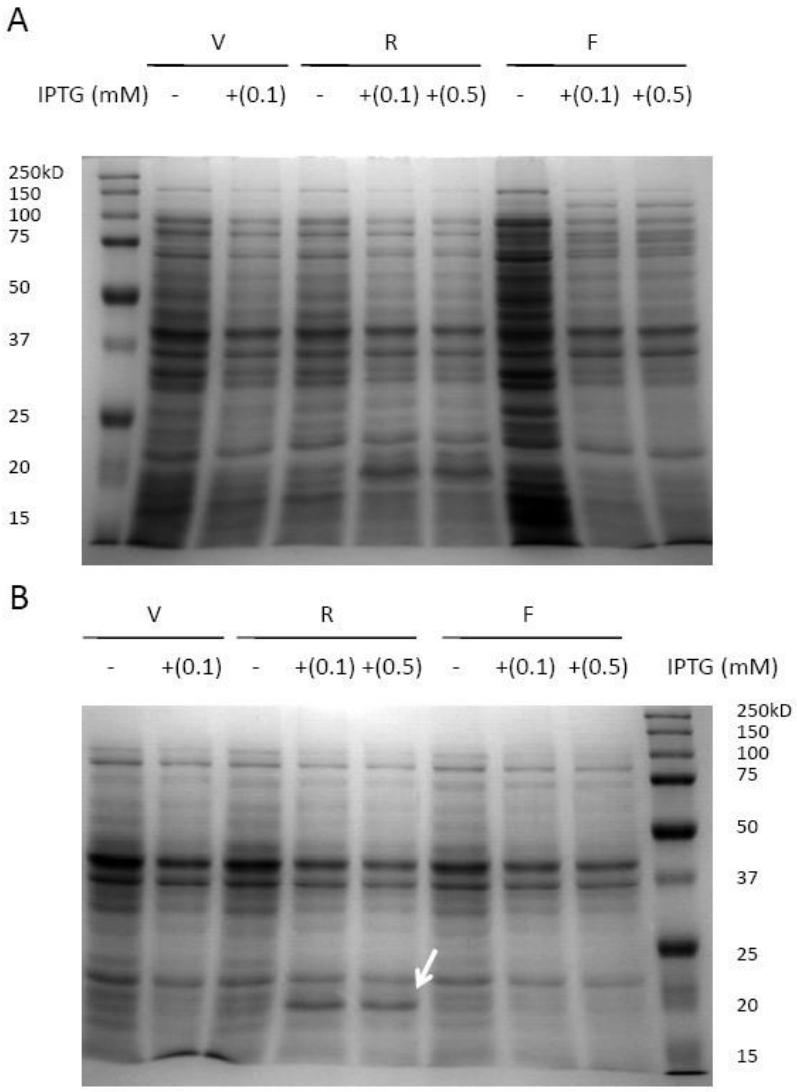


Figure 3.10 SDS-PAGE analysis of protein expression of His-AtRad5a and His-AtRad5aRING in a small scale of bacterial cells. BL21 (DE3)-RIL cells carrying pET28c-AtRAD5a or pET28c-AtRAD5a-RING were induced using two different concentrations of IPTG and incubated overnight. Cells lysate (A) and soluble fraction (B) were prepared and subjected to SDS-PAGE. Samples and IPTG concentrations were indicated on the upper panel. V, R and F refer to samples of pET28c vector alone, pET28-AtRAD5a-RING and pET28-AtRAD5a. White arrow shows the position of His-AtRad5aRING band.

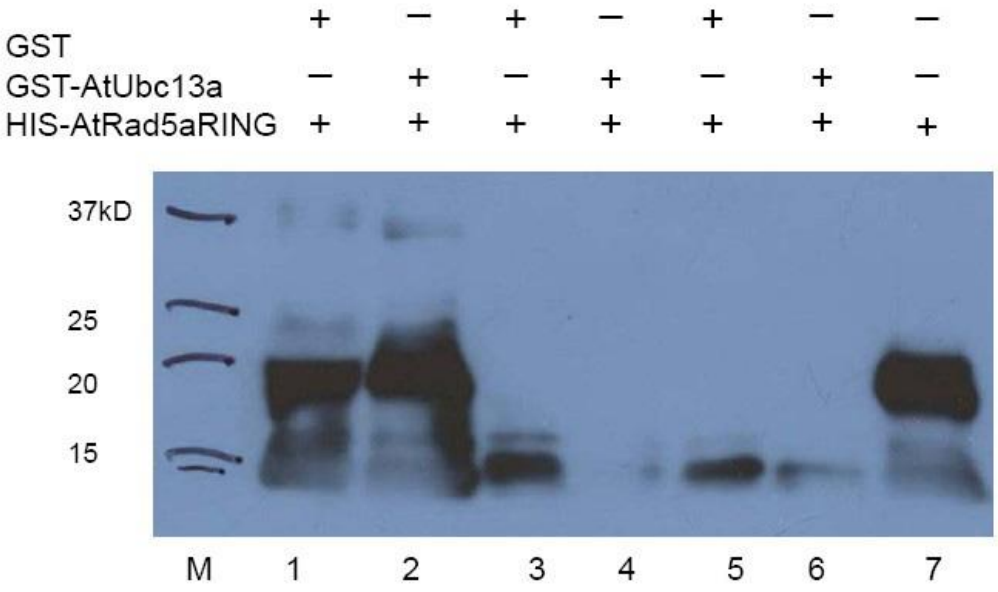


Figure 3.11 Western blotting analysis of GST-pull down assay. Purified GST or GST-AtUbc13a was added to GST microspin columns. After incubation, columns were spun and washed. Then, purified His-AtRad5aRING was added and incubated. After washing, the columns were eluted with reduced glutathione and subjected to SDS-PAGE and western blotting analysis. Proteins were transferred to the PVDF membrane and detected with an antibody against the His tag. Lanes 1 and 2 shows the flow-through solution after washing. Lanes 3 and 4 shows the first elution solutions. Lanes 5 and 6 shows the second elution solutions.

3.6 Complementation of Yeast *rad5* Mutant by *AtRAD5a*

One of the remarkable phenotypes of *rad5* mutant is the increased sensitivity to many DNA-damaging agents, which can be assessed by a gradient plate assay. To investigate whether *AtRAD5a* is able to functionally complement the budding yeast *rad5* mutant, a plasmid expressing *AtRAD5a* was used to transform the yeast *rad5* mutant cells. In the gradient plate containing MMS, *rad5a* mutant showed slow growth compared to the wild-type cells. Expression of *AtRAD5a* from the yeast two-hybrid plasmid could not rescue the *rad5* mutants from killing by MMS (Fig. 3.12).

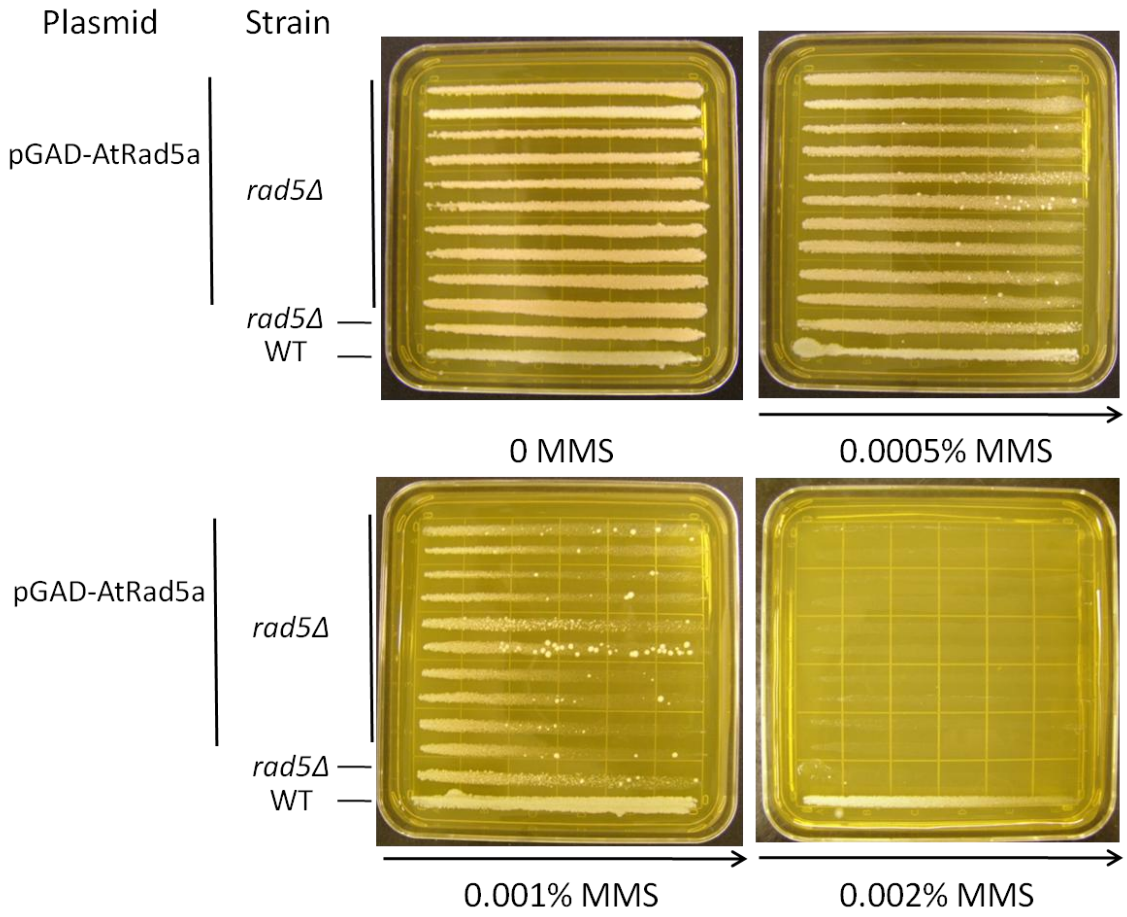


Figure 3.12 *AtRAD5a* could not complement the yeast rad5 mutant. The YPD control and YPD containing different concentrations of MMS gradient plates were incubated at 30 ℃ for 3 days. Arrow points to gradually increasing MMS concentrations. The strains and plasmid used were listed on the left side of the figure. WT strain: the bottom line of the plate. *rad5*Δ strain: the last second line of the plate. *rad5*Δ strain with plasmid pGAD-AtRad5a transformed: the other lines on plate except the last two lines.

4. DISCUSSION

In this study, we investigated the genetic interactions between *AtRAD5a* and *AtREV3* in the protection of plant seedlings from growth inhibition by various DNA damaging agents. Although we were able to observe previously reported phenotypes on each of the single mutants, the primary focus in this study was to examine genetic interactions of the two genes by assessing phenotypes of the *rad5 rev3* double mutant plants in response to DNA damaging agents at the doses when neither single mutant displayed apparent phenotypic alterations. This investigation surprisingly revealed three possible modes of genetic interactions between pathways represented by *AtRAD5a* and *AtREV3*.

4.1 Two Alternative DDT Pathways Represented by *AtRAD5a* and *AtREV3*

The primary objective of this investigation was to see whether plants, like budding yeast, possess two alternative mechanisms of DDT to bypass replication-blocking lesions. By using a classical agent MMS that induces replication-blocking lesions and activates S-phase checkpoint (Paulovich and Hartwell, 1995), we were able to show that at MMS doses that did not inhibit the growth of either single mutant, the growth of the *rad5a rev3* double mutant was severely inhibited; quantitative analysis indicated that the effects of the two mutations are synergistic, suggesting that plant cells can utilize either Rev3- or Rad5A-mediated pathways to bypass MMS-induced lesions. However, the biological consequences are expected to be different: Rev3-mediated TLS is expected to cause increased mutagenesis, whereas Rad5-mediated lesion bypass is deemed error-free. Whether this is indeed the case awaits the development of convenient plant mutagenesis assays for further investigation. Nevertheless, to our knowledge, this would be the first evidence for the existence of two-branch DDT in a multicellular organism.

Is the two-branch DDT in higher eukaryotes regulated by sequential ubiquitinations of

PCNA, as reported in yeast? In mammalian cells, it is generally accepted that Rad18 is required for PCNA monoubiquitination, which in turn recruits Y-family polymerases (Bienko et al., 2005; Kannouche et al., 2004). A number of reports have addressed the *in vitro* and *in vivo* activities of plant TLS polymerases (Anderson et al., 2008; Curtis and Hays, 2007; Garcia-Ortiz et al., 2004; Sakamoto et al., 2003; Santiago et al., 2006; Takahashi et al., 2005); however, the requirement of PCNA ubiquitination for their *in vivo* activity has not been reported, although there is an indirect observation that AtPCNA2 can stimulate AtPolη activity in yeast cells (Anderson et al., 2008).

Arabidopsis genes potentially required for PCNA polyubiquitination have been identified, including *UBC13*, *UEV1* and *RAD5*. Because *AtUBC13* and possibly *AtUEV1* genes are multifunctional and their null mutations may affect other aspects of plant growth (data not shown), here we utilized *AtRAD5a* to represent the error-free DDT branch and demonstrated that *rad5a* is indeed synergistic with *rev3* for MMS sensitivity. The yeast Rad5 protein contains a SWI/SNF family domain and a conserved ATPase sequence (Johnson et al., 1992) as well as a RING-finger motif, which is required to interact with Ubc13 (Ulrich, 2003). Purified Rad5 protein indeed possesses ATPase (Gangavarapu et al., 2006) and helicase activities to promotes replication fork regression (Blastyak et al., 2007), and a E3 ligase activity to promote Lys63-linked polyubiquitination (Parker and Ulrich, 2009); both activities appear to be required for the error-free mode of DDT. While this study provides yet the strongest possible evidence for the two-branch DDT model in plants to date, we have not been able to demonstrate the anticipated AtRad5a-AtUbc13 physical interaction. The negative results obtained from yeast two-hybrid and GST-pull down assays do not necessarily exclude the possibility of interaction between AtRad5a and AtUbc13. For AtRad5a-RING, the negative result may be due to the improper folding when it is expressed, since the flanking sequences besides RING-finger domain play an important role

on protein function. For the full-length AtRad5a, its large size may cause difficulty in protein expression and folding in yeast or bacterial cells. Actually the physical interaction between yeast Ubc13 and Rad5 has only been demonstrated by a yeast two-hybrid assay (Ulrich and Jentsch, 2000), and this interaction is very weak and could only be detected in one orientation (data not shown).

4.2 Differential Response of *rad5a* and *rev3* Mutants to UV and UV-mimetic Agents

Plants as sessile organisms are constantly exposed to sunlight and must have developed sophisticated mechanisms to protect against UV-induced DNA damage. It is clear from this study that plants mainly utilize the TLS mode of DDT to bypass UV-induced lesions. Under our experimental conditions, we were unable to observe an increased sensitivity of *rad5a-3* mutant to UV irradiation; however, under multiple exposure conditions, Rad5a may provide moderate protection only when Rev3 is inactivated. To avoid the concern that UV-induced DNA damage may be undermined by the powerful photoreactivation in plants, we examined the plant response to a UV-mimetic agent 4NQO that induces bulky lesions not repaired by photoreactivation (Friedberg et al., 2006). This study revealed that the *rad5a* single mutant is more sensitive to 4NQO than wild-type but less sensitive than the *rev3* mutant, and the two mutations show a typical additive effect. It appears to indicate that Rad5a specifically acts on persistent lesions, while TLS can act on transient replication-blocking lesions. An alternative explanation would be that Rad5a does not act on replication-blocking lesions immediately but instead on the secondary products when other repair/bypass means have failed.

4.3 Mechanisms of Tolerance to Crosslinking Agents by AtRad5a and AtRev3

Despite the claim that cisplatin and MMC may preferentially induce different types of crosslinks, it is generally accepted that interstrand crosslinks (ICLs) induced by these agents

have the most biological effects (Lehoczky et al., 2007). In yeast, the lesion bypass of ICLs by Polζ occurs in G1 and this process does not involve other members of DDT (Sarkar et al., 2006); hence, it is not considered a *RAD6*-dependent DDT mechanism. Indeed, despite the fact that the yeast *rad5* mutant is much more sensitive to MMS, UV and 4NQO than *rev3*, it is not sensitive to cisplatin and MMC under our experimental conditions. Although *rad5* is additive to *rev3* with respect to cisplatin and MMC sensitivity, it is probably due to the fact that Rad5 participates in the repair of DSBs independent of its activity in error-free DDT (Chen et al., 2005). Interestingly AtRad5a is also reported to be involved in DSB-induced HR (Chen et al., 2008). Since HR is required for the repair of ICLs at G2 (Lehoczky et al., 2007) while Polζ is probably involved in bypassing ICLs in G1, we conclude that the strong additive effects between plant *rad5a* and *rev3* for ICL-induced damage is due to their distinct involvement in the repair and tolerance of ICLs, respectively. Nevertheless, we cannot rule out the possibility that plant Rad5a and Rev3 are involved in bypassing intra-strand crosslinks induced by cisplatin and MMC via *RAD6*-dependent DDT.

4.4 DNA Damage Treatment by MMS Enhances Endoreduplication in *rad5a rev3* Double Mutant

It is known that endoreduplication can be induced or enhanced by various factors in both animals and plants (Lee et al., 2009). Little is known concerning the relationship between DNA damage and endoreduplication in plants. Our results show that DNA damage by low dosage MMS treatment results in an increased level of endopolyploidy in the *rad5a-3 rev3-2* double mutant. Conceivably, more DNA damage lesions are present in the *rad5-3 rev3-2* double mutant since the lesions are not repaired or bypassed during DNA replication. It is possible that such lesions may trigger an unknown mechanism to inhibit mitosis, leading to an increased nuclear

DNA content. According to the view that cyclin-dependent kinases (CDKs) regulate both endoreduplication and mitosis (Larkins et al., 2001), the simplest explanation is that the lesions from DNA damage treatment result in incomplete DNA replication and consequently in the inhibition of certain CDKs required for the entry into mitosis. At the same time, these results also imply that despite the presence of the lesions, DNA replication can proceed for one or more rounds without the subsequent mitosis.

4.5 Other Rad5-like Proteins in Arabidopsis

Inactivation of *AtRAD5a* results in an increased sensitivity to DNA-damaging agents, but *AtRAD5b* seems dispensable since its mutant has no such phenotypes. However, the possibility of AtRad5b involvement in DNA repair cannot be excluded. The biochemical studies about these two proteins are still limited, compared to their analogues in yeast and mammalian cells. A very recent study shows that the two mammalian *RAD5* homologs have different responses to UV and MMS treatments (Lin et al., 2011). Although only *AtRAD5a* was identified as a gene related to DDT in Arabidopsis, there are additional candidates yet to be characterized in the RAD5-like gene family. In addition to *AtRAD5a* and *AtRAD5b*, Arabidopsis has 10 other genes whose products share varying degrees of similarities to Rad5 in budding yeast. For example, *At5g05130* has a lower level of similarity to yeast Rad5 than AtRad5a and AtRad5b, and also all the domains of Rad5 are conserved in its protein sequence. However, currently there is no biochemical or biological information on this gene product. Thus, functional studies on each member of this Rad5-like protein family in Arabidopsis will be useful.

4.6 Rad5a, Rev1 and Polζ May be Cooperatively Involved in Homologous Recombination Repair

Homologous recombination (HR) is also a key pathway for the repair of different DNA

damages, especially DSBs. Previous studies suggest that Polζ may play an important role in HR, since deficiencies of this DNA polymerase are associated with IR sensitivity, and high frequencies of chromosomal aberrations (Okada et al., 2005; Wittschieben et al., 2006). These phenotypes are similar to those of HR repair deficient mutants. Very recently, a study shows that Rev1 and Polζ forming a complex promote HR repair in mammalian cells. The cells lacking Rev3 were hypersensitive to agents causing DSBs (Sharma et al., 2011). Surprisingly, cells depleted of Rad18 did not show similar deficiencies, suggesting that Rev1 and Polζ operate in HR repair pathway independent of their functions of TLS. In Arabidopsis, no evidence at the moment indicates that AtRev1, AtRev3 and AtRev7 are involved in HR. However, in yeast, Rad5 is able to interact with Rev1 (Pages et al., 2008) suggesting these proteins may work together. Combining with data that AtRad5a is also reported to be involved in DSB-induced HR (Chen et al., 2008), AtRad5a, AtRev1 and Polζ maybe cooperatively involve in homologous recombination repair. However, it needs more investigation.

4.7 Conclusions

This study made three major discoveries. Firstly, using MMS as a classical DNA damaging agent that primarily induces replication-blocking lesions and S-phase checkpoint, we were able to demonstrate that *AtRAD5a* and *AtREV3* constitute two alternative DNA-damage tolerance pathways reminiscent of the yeast two-branch DDT/PRR pathway. Given the high degree of amino acid sequence similarity between yeast and plant Rad5 and Rev3 proteins, it is conceivable that two modes of DDT through sequential ubiquitination of PCNA are conserved in plants. Secondly, this study reveals three distinct types of genetic interactions between *AtRAD5a* and *AtREV3* in response to different DNA-damaging agents. While *rad5a* and *rev3* are synergistic with respect to MMS-induced DNA damage, they are barely additive to UV and

bulky adducts. On the other hand, although *rad5a* and *rev3* mutations confer strong additive effects to killing by ICLs, we argue based on current knowledge that this is unlikely due to the activity of DDT mediated by PCNA ubiquitination. Thirdly, we demonstrated that the relative contributions of Rev3 and Rad5 in Arabidopsis are rather different from budding yeast. AtRev3 appears to play a major role in tolerance to all types of DNA-damaging agents, particularly UV irradiation, bulky adducts and ICLs, suggesting that TLS is a preferred mechanism of lesion bypass in plants. In contrast, the budding yeast *rad5* null mutant is much more sensitive to MMS, UV and 4NQO than *rev3*, indicating that in yeast cells an error-free bypass is favored over TLS in the presence of the above DNA-damaging agents. In addition, we surprisingly found that treatment with MMS leads to a clear increase in the nuclear DNA ploidy level.

5. REFERENCES

Acharya, N., Haracska, L., Johnson, R.E., Unk, I., Prakash, S., and Prakash, L. (2005). Complex formation of yeast Rev1 and Rev7 proteins: a novel role for the polymerase-associated domain. Mol Cell Biol *25*, 9734-9740.

AGI (2000). Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature *408*, 796-815.

Ahmad, M., and Cashmore, A.R. (1993). HY4 gene of A. thaliana encodes a protein with characteristics of a blue-light photoreceptor. Nature *366*, 162-166.

Ahne, F., Jha, B., and Eckardt-Schupp, F. (1997). The RAD5 gene product is involved in the avoidance of non-homologous end-joining of DNA double strand breaks in the yeast Saccharomyces cerevisiae. Nucleic Acids Res *25*, 743-749.

Andersen, P.L., Xu, F., and Xiao, W. (2008). Eukaryotic DNA damage tolerance and translesion synthesis through covalent modifications of PCNA. Cell Res *18*, 162-173.

Andersen, P.L., Zhou, H., Pastushok, L., Moraes, T., McKenna, S., Ziola, B., Ellison, M.J., Dixit, V.M., and Xiao, W. (2005). Distinct regulation of Ubc13 functions by the two ubiquitin-conjugating enzyme variants Mms2 and Uev1A. J Cell Biol *170*, 745-755.

Anderson, H.J., Vonarx, E.J., Pastushok, L., Nakagawa, M., Katafuchi, A., Gruz, P., Di Rubbo, A., Grice, D.M., Osmond, M.J., Sakamoto, A.N., *et al.* (2008). Arabidopsis thaliana Y-family DNA polymerase eta catalyses translesion synthesis and interacts functionally with PCNA2. Plant J *55*, 895-908.

Baarends, W.M., Wassenaar, E., Hoogerbrugge, J.W., van Cappellen, G., Roest, H.P., Vreeburg, J., Ooms, M., Hoeijmakers, J.H., and Grootegoed, J.A. (2003). Loss of HR6B ubiquitin-conjugating activity results in damaged synaptonemal complex structure and increased crossing-over frequency during the male meiotic prophase. Mol Cell Biol *23*, 1151-1162.

Bailly, V., Lamb, J., Sung, P., Prakash, S., and Prakash, L. (1994). Specific complex formation between yeast RAD6 and RAD18 proteins: a potential mechanism for targeting RAD6 ubiquitin-conjugating activity to DNA damage sites. Genes Dev *8*, 811-820.

Bailly, V., Lauder, S., Prakash, S., and Prakash, L. (1997). Yeast DNA repair proteins Rad6 and Rad18 form a heterodimer that has ubiquitin conjugating, DNA binding, and ATP hydrolytic activities. J Biol Chem *272*, 23360-23365.

Barbour, L., Ball, L.G., Zhang, K., and Xiao, W. (2006). DNA damage checkpoints are involved in postreplication repair. Genetics *174*, 1789-1800.

Barow, M. (2006). Endopolyploidy in seed plants. Bioessays *28*, 271-281.

Bartel, B., Wunning, I., and Varshavsky, A. (1990). The recognition component of the N-end rule

pathway. EMBO J *9*, 3179-3189.

Bienko, M., Green, C.M., Crosetto, N., Rudolf, F., Zapart, G., Coull, B., Kannouche, P., Wider, G., Peter, M., Lehmann, A.R., *et al.* (2005). Ubiquitin-binding domains in Y-family polymerases regulate translesion synthesis. Science *310*, 1821-1824.

Blastyak, A., Pinter, L., Unk, I., Prakash, L., Prakash, S., and Haracska, L. (2007). Yeast Rad5 protein required for postreplication repair has a DNA helicase activity specific for replication fork regression. Mol Cell *28*, 167-175.

Boorstein, R.J., Hilbert, T.P., Cunningham, R.P., and Teebor, G.W. (1990). Formation and stability of repairable pyrimidine photohydrates in DNA. Biochemistry *29*, 10455-10460.

Britt, A.B. (1999). Molecular genetics of DNA repair in higher plants. Trends Plant Sci *4*, 20-25.

Britt, A.B., Chen, J.J., Wykoff, D., and Mitchell, D. (1993). A UV-sensitive mutant of Arabidopsis defective in the repair of pyrimidine-pyrimidinone(6-4) dimers. Science *261*, 1571-1574.

Broomfield, S., Chow, B.L., and Xiao, W. (1998). MMS2, encoding a ubiquitin-conjugating-enzyme-like protein, is a member of the yeast error-free postreplication repair pathway. Proc Natl Acad Sci U S A *95*, 5678-5683.

Broomfield, S., Hryciw, T., and Xiao, W. (2001). DNA postreplication repair and mutagenesis in Saccharomyces cerevisiae. Mutat Res *486*, 167-184.

Brown, M., Zhu, Y., Hemmingsen, S.M., and Xiao, W. (2002). Structural and functional conservation of error-free DNA postreplication repair in Schizosaccharomyces pombe. DNA Repair (Amst) *1*, 869-880.

Brusky, J., Zhu, Y., and Xiao, W. (2000). UBC13, a DNA-damage-inducible gene, is a member of the error-free postreplication repair pathway in Saccharomyces cerevisiae. Curr Genet *37*, 168-174.

Cao, Y., Dai, Y., Cui, S., and Ma, L. (2008). Histone H2B monoubiquitination in the chromatin of FLOWERING LOCUS C regulates flowering time in Arabidopsis. Plant Cell *20*, 2586-2602.

Carlile, C.M., Pickart, C.M., Matunis, M.J., and Cohen, R.E. (2009). Synthesis of free and proliferating cell nuclear antigen-bound polyubiquitin chains by the RING E3 ubiquitin ligase Rad5. J Biol Chem *284*, 29326-29334.

Chen, I.P., Mannuss, A., Orel, N., Heitzeberg, F., and Puchta, H. (2008). A homolog of ScRAD5 is involved in DNA repair and homologous recombination in Arabidopsis. Plant Physiol *146*, 1786-1796.

Chen, S., Davies, A.A., Sagan, D., and Ulrich, H.D. (2005). The RING finger ATPase Rad5p of Saccharomyces cerevisiae contributes to DNA double-strand break repair in a ubiquitin-independent manner. Nucleic Acids Res *33*, 5878-5886.

Curtis, M.J., and Hays, J.B. (2007). Tolerance of dividing cells to replication stress in UVB-irradiated Arabidopsis roots: requirements for DNA translesion polymerases eta and zeta. DNA Repair (Amst) *6*, 1341-1358.

Denver, D.R., Swenson, S.L., and Lynch, M. (2003). An evolutionary analysis of the helix-hairpin-helix superfamily of DNA repair glycosylases. Mol Biol Evol *20*, 1603-1611.

Deshaies, R.J., and Joazeiro, C.A. (2009). RING domain E3 ubiquitin ligases. Annu Rev Biochem *78*, 399-434.

Diaz, M., Watson, N.B., Turkington, G., Verkoczy, L.K., Klinman, N.R., and McGregor, W.G. (2003). Decreased frequency and highly aberrant spectrum of ultraviolet-induced mutations in the hprt gene of mouse fibroblasts expressing antisense RNA to DNA polymerase zeta. Mol Cancer Res *1*, 836-847.

Douki, T., and J. Cadet. (2003). Formation of the spore photoproduct and other dimeric lessions between adjacent pyrimidines in UVC-irradiated dry DNA. Photochem Photobiol Sci *2*, 433-436.

Douki, T., Laporte, G., and Cadet, J. (2003). Inter-strand photoproducts are produced in high yield within A-DNA exposed to UVC radiation. Nucleic Acids Res *31*, 3134-3142.

Douziech, M., Coin, F., Chipoulet, J.M., Arai, Y., Ohkuma, Y., Egly, J.M., and Coulombe, B. (2000). Mechanism of promoter melting by the xeroderma pigmentosum complementation group B helicase of transcription factor IIH revealed by protein-DNA photo-cross-linking. Mol Cell Biol *20*, 8168-8177.

Eastman, A. (1987). The formation, isolation and characterization of DNA adducts produced by anticancer platinum complexes. Pharmacol Ther *34*, 155-166.

Eddins, M.J., Carlile, C.M., Gomez, K.M., Pickart, C.M., and Wolberger, C. (2006). Mms2-Ubc13 covalently bound to ubiquitin reveals the structural basis of linkage-specific polyubiquitin chain formation. Nat Struct Mol Biol *13*, 915-920.

Edwards, K., Johnstone, C., and Thompson, C. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res *19*, 1349.

Finley, D., Ozkaynak, E., and Varshavsky, A. (1987). The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation, and other stresses. Cell *48*, 1035-1046.

Freemont, P.S., Hanson, I.M., and Trowsdale, J. (1991). A novel cysteine-rich sequence motif. Cell *64*, 483-484.

Friedberg, E.C., Walker, G.C., Siede, W., Wood, R.D., Schultz, R.A., and Ellenberger, T. (2006). DNA Repair and Mutagenesis, 2nd Edition (Washington, D.C., ASM Press).

Friedberg, E.C., Walker, G.C., Siede, W., Wood, R.D., Schultz, R.A. and Ellenberger, T. , ed. (2006). (ASM Press, Washington, D. C).

Galbraith, D.W., Harkins, K.R., and Knapp, S. (1991). Systemic Endopolyploidy in Arabidopsis thaliana. Plant Physiol *96*, 985-989.

Galiegue-Zouitina, S., Bailleul, B., Ginot, Y.M., Perly, B., Vigny, P., and Loucheux-Lefebvre, M.H. (1986). N2-guanyl and N6-adenyl arylation of chicken erythrocyte DNA by the ultimate carcinogen of 4-nitroquinoline 1-oxide. Cancer Res *46*, 1858-1863.

Galiegue-Zouitina, S., Bailleul, B., and Loucheux-Lefebvre, M.H. (1985). Adducts from in vivo action of the carcinogen 4-hydroxyaminoquinoline 1-oxide in rats and from in vitro reaction of 4-acetoxyaminoquinoline 1-oxide with DNA and polynucleotides. Cancer Res *45*, 520-525.

Gangavarapu, V., Haracska, L., Unk, I., Johnson, R.E., Prakash, S., and Prakash, L. (2006). Mms2-Ubc13-dependent and -independent roles of Rad5 ubiquitin ligase in postreplication repair and translesion DNA synthesis in Saccharomyces cerevisiae. Mol Cell Biol *26*, 7783-7790.

Garcia-Ortiz, M.V., Ariza, R.R., Hoffman, P.D., Hays, J.B., and Roldan-Arjona, T. (2004). Arabidopsis thaliana AtPOLK encodes a DinB-like DNA polymerase that extends mispaired primer termini and is highly expressed in a variety of tissues. Plant J *39*, 84-97.

Garg, P., and Burgers, P.M. (2005). Ubiquitinated proliferating cell nuclear antigen activates translesion DNA polymerases eta and REV1. Proc Natl Acad Sci U S A *102*, 18361-18366.

Gibbs, P.E., McGregor, W.G., Maher, V.M., Nisson, P., and Lawrence, C.W. (1998). A human homolog of the Saccharomyces cerevisiae REV3 gene, which encodes the catalytic subunit of DNA polymerase zeta. Proc Natl Acad Sci U S A *95*, 6876-6880.

Gibbs, P.E., Wang, X.D., Li, Z., McManus, T.P., McGregor, W.G., Lawrence, C.W., and Maher, V.M. (2000). The function of the human homolog of Saccharomyces cerevisiae REV1 is required for mutagenesis induced by UV light. Proc Natl Acad Sci U S A *97*, 4186-4191.

Gu, X., Jiang, D., Wang, Y., Bachmair, A., and He, Y. (2009). Repression of the floral transition via histone H2B monoubiquitination. Plant J *57*, 522-533.

Guo, C., Fischhaber, P.L., Luk-Paszyc, M.J., Masuda, Y., Zhou, J., Kamiya, K., Kisker, C., and Friedberg, E.C. (2003). Mouse Rev1 protein interacts with multiple DNA polymerases involved in translesion DNA synthesis. EMBO J *22*, 6621-6630.

Guo, C., Sonoda, E., Tang, T.S., Parker, J.L., Bielen, A.B., Takeda, S., Ulrich, H.D., and Friedberg, E.C. (2006a). REV1 protein interacts with PCNA: significance of the REV1 BRCT domain in vitro and in vivo. Mol Cell *23*, 265-271.

Guo, C., Tang, T.S., Bienko, M., Parker, J.L., Bielen, A.B., Sonoda, E., Takeda, S., Ulrich, H.D., Dikic, I., and Friedberg, E.C. (2006b). Ubiquitin-binding motifs in REV1 protein are required for its role in the tolerance of DNA damage. Mol Cell Biol *26*, 8892-8900.

Haglund, K., and Dikic, I. (2005). Ubiquitylation and cell signaling. EMBO J *24*, 3353-3359.

Hays, J.B. (2002). Arabidopsis thaliana, a versatile model system for study of eukaryotic

genome-maintenance functions. DNA Repair (Amst) *1*, 579-600.

Hill, J., Donald, K.A., and Griffiths, D.E. (1991). DMSO-enhanced whole cell yeast transformation. Nucleic Acids Res *19*, 5791.

Hoege, C., Pfander, B., Moldovan, G.L., Pyrowolakis, G., and Jentsch, S. (2002). RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. Nature *419*, 135-141.

Hoffman, P.D., Leonard, J.M., Lindberg, G.E., Bollmann, S.R., and Hays, J.B. (2004). Rapid accumulation of mutations during seed-to-seed propagation of mismatch-repair-defective Arabidopsis. Genes Dev *18*, 2676-2685.

Hofmann, R.M., and Pickart, C.M. (1999). Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. Cell *96*, 645-653.

Hofmann, R.M., and Pickart, C.M. (2001). In vitro assembly and recognition of Lys-63 polyubiquitin chains. J Biol Chem *276*, 27936-27943.

Hotton, S.K., and Callis, J. (2008). Regulation of cullin RING ligases. Annu Rev Plant Biol *59*, 467-489.

Iyer, L.M., Babu, M.M., and Aravind, L. (2006). The HIRAN domain and recruitment of chromatin remodeling and repair activities to damaged DNA. Cell Cycle *5*, 775-782.

James, P., Halladay, J., and Craig, E.A. (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics *144*, 1425-1436.

Jentsch, S., McGrath, J.P., and Varshavsky, A. (1987). The yeast DNA repair gene RAD6 encodes a ubiquitin-conjugating enzyme. Nature *329*, 131-134.

Johnson, R.E., Henderson, S.T., Petes, T.D., Prakash, S., Bankmann, M., and Prakash, L. (1992). Saccharomyces cerevisiae RAD5-encoded DNA repair protein contains DNA helicase and zinc-binding sequence motifs and affects the stability of simple repetitive sequences in the genome. Mol Cell Biol *12*, 3807-3818.

Johnson, R.E., Kondratick, C.M., Prakash, S., and Prakash, L. (1999a). hRAD30 mutations in the variant form of xeroderma pigmentosum. Science *285*, 263-265.

Johnson, R.E., Prakash, S., and Prakash, L. (1994). Yeast DNA repair protein RAD5 that promotes instability of simple repetitive sequences is a DNA-dependent ATPase. J Biol Chem *269*, 28259-28262.

Johnson, R.E., Prakash, S., and Prakash, L. (1999b). Efficient bypass of a thymine-thymine dimer by yeast DNA polymerase, Poleta. Science *283*, 1001-1004.

Jones, J.S., Weber, S., and Prakash, L. (1988). The Saccharomyces cerevisiae RAD18 gene encodes a protein that contains potential zinc finger domains for nucleic acid binding and a

putative nucleotide binding sequence. Nucleic Acids Res *16*, 7119-7131.

Kannouche, P.L., Wing, J., and Lehmann, A.R. (2004). Interaction of human DNA polymerase eta with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage. Mol Cell *14*, 491-500.

Koken, M., Reynolds, P., Bootsma, D., Hoeijmakers, J., Prakash, S., and Prakash, L. (1991a). Dhr6, a Drosophila homolog of the yeast DNA-repair gene RAD6. Proc Natl Acad Sci U S A *88*, 3832-3836.

Koken, M.H., Reynolds, P., Jaspers-Dekker, I., Prakash, L., Prakash, S., Bootsma, D., and Hoeijmakers, J.H. (1991b). Structural and functional conservation of two human homologs of the yeast DNA repair gene RAD6. Proc Natl Acad Sci U S A *88*, 8865-8869.

Kraft, E., Stone, S.L., Ma, L., Su, N., Gao, Y., Lau, O.S., Deng, X.W., and Callis, J. (2005). Genome analysis and functional characterization of the E2 and RING-type E3 ligase ubiquitination enzymes of Arabidopsis. Plant Physiol *139*, 1597-1611.

Krijger, P.H., Lee, K.Y., Wit, N., van den Berk, P.C., Wu, X., Roest, H.P., Maas, A., Ding, H., Hoeijmakers, J.H., Myung, K., *et al.* (2011). HLTF and SHPRH are not essential for PCNA polyubiquitination, survival and somatic hypermutation: existence of an alternative E3 ligase. DNA Repair (Amst) *10*, 438-444.

Kunz, B.A., and Haynes, R.H. (1981). Phenomenology and genetic control of mitotic recombination in yeast. Annu Rev Genet *15*, 57-89.

Kunz, B.A., Kang, X.L., and Kohalmi, L. (1991). The yeast rad18 mutator specifically increases G.C----T.A transversions without reducing correction of G-A or C-T mismatches to G.C pairs. Mol Cell Biol *11*, 218-225.

Kunz, B.A., and Xiao, W. (2007). DNA damage tolerance in plants via translesion synthesis. Genes, Genomes & Genomics *1*, 89-99.

Kunz, B.A.a.X., W. (2007). DNA damage tolerance in plants via translesion synthesis. Genes, Genomes & Genomics *1*, 89-99.

Larimer, F.W., Perry, J.R., and Hardigree, A.A. (1989). The REV1 gene of Saccharomyces cerevisiae: isolation, sequence, and functional analysis. J Bacteriol *171*, 230-237.

Larkins, B.A., Dilkes, B.P., Dante, R.A., Coelho, C.M., Woo, Y.M., and Liu, Y. (2001). Investigating the hows and whys of DNA endoreduplication. J Exp Bot *52*, 183-192.

Lawley, P.D., and Phillips, D.H. (1996). DNA adducts from chemotherapeutic agents. Mutat Res *355*, 13-40.

Lawrence, C.W. (2002). Cellular roles of DNA polymerase zeta and Rev1 protein. DNA Repair (Amst) *1*, 425-435.

Lawrence, C.W., Das, G., and Christensen, R.B. (1985a). REV7, a new gene concerned with UV mutagenesis in yeast. Mol Gen Genet *200*, 80-85.

Lawrence, C.W., Nisson, P.E., and Christensen, R.B. (1985b). UV and chemical mutagenesis in rev7 mutants of yeast. Mol Gen Genet *200*, 86-91.

Lee, H.O., Davidson, J.M., and Duronio, R.J. (2009). Endoreplication: polyploidy with purpose. Genes Dev *23*, 2461-2477.

Lehoczky, P., McHugh, P.J., and Chovanec, M. (2007). DNA interstrand cross-link repair in Saccharomyces cerevisiae. FEMS Microbiol Rev *31*, 109-133.

Lemontt, J.F. (1971a). Mutants of yeast defective in mutation induced by ultraviolet light. Genetics *68*, 21-33.

Lemontt, J.F. (1971b). Pathways of ultraviolet mutability in Saccharomyces cerevisiae. II. The effect of rev genes on recombination. Mutat Res *13*, 319-326.

Li, W., and Schmidt, W. (2010). A lysine-63-linked ubiquitin chain-forming conjugase, UBC13, promotes the developmental responses to iron deficiency in Arabidopsis roots. Plant J *62*, 330-343.

Lin, C., Yang, H., Guo, H., Mockler, T., Chen, J., and Cashmore, A.R. (1998). Enhancement of blue-light sensitivity of Arabidopsis seedlings by a blue light receptor cryptochrome 2. Proc Natl Acad Sci U S A *95*, 2686-2690.

Lin, J.R., Zeman, M.K., Chen, J.Y., Yee, M.C., and Cimprich, K.A. (2011). SHPRH and HLTF act in a damage-specific manner to coordinate different forms of postreplication repair and prevent mutagenesis. Mol Cell *42*, 237-249.

Lyakhovich, A., and Shekhar, M.P. (2004). RAD6B overexpression confers chemoresistance: RAD6 expression during cell cycle and its redistribution to chromatin during DNA damage-induced response. Oncogene *23*, 3097-3106.

Magana-Schwencke, N., Henriques, J.A., Chanet, R., and Moustacchi, E. (1982). The fate of 8-methoxypsoralen photoinduced crosslinks in nuclear and mitochondrial yeast DNA: comparison of wild-type and repair-deficient strains. Proc Natl Acad Sci U S A *79*, 1722-1726.

McDonald, J.P., Levine, A.S., and Woodgate, R. (1997). The Saccharomyces cerevisiae RAD30 gene, a homologue of Escherichia coli dinB and umuC, is DNA damage inducible and functions in a novel error-free postreplication repair mechanism. Genetics *147*, 1557-1568.

McKenna, S., Spyracopoulos, L., Moraes, T., Pastushok, L., Ptak, C., Xiao, W., and Ellison, M.J. (2001). Noncovalent interaction between ubiquitin and the human DNA repair protein Mms2 is required for Ubc13-mediated polyubiquitination. J Biol Chem *276*, 40120-40126.

Menichini, P., Fronza, G., Tornaletti, S., Galiegue-Zouitina, S., Bailleul, B., Loucheux-Lefebvre, M.H., Abbondandolo, A., and Pedrini, A.M. (1989). In vitro DNA modification by the ultimate

carcinogen of 4-nitroquinoline-1-oxide: influence of superhelicity. Carcinogenesis *10*, 1589-1593.

Miller, E.C., and J. A. Miller. (1947). The presence and significance of bound aminoazo dyes in the livers of rats fed *p*-dimethylaminoazobenzene. Cancer Res *7*, 468-480.

Mitchell, D.L., and R. S. Nairn (1989). The biology of the (6-4) photoproduct. Photochem Photobiol *49*, 805-819.

Mitchell, D.L., Jen, J., and Cleaver, J.E. (1992). Sequence specificity of cyclobutane pyrimidine dimers in DNA treated with solar (ultraviolet B) radiation. Nucleic Acids Res *20*, 225-229.

Moldovan, G.L., Pfander, B., and Jentsch, S. (2007). PCNA, the maestro of the replication fork. Cell *129*, 665-679.

Montelone, B.A., Prakash, S., and Prakash, L. (1981). Recombination and mutagenesis in rad6 mutants of Saccharomyces cerevisiae: evidence for multiple functions of the RAD6 gene. Mol Gen Genet *184*, 410-415.

Morrison, A., Christensen, R.B., Alley, J., Beck, A.K., Bernstine, E.G., Lemontt, J.F., and Lawrence, C.W. (1989). REV3, a Saccharomyces cerevisiae gene whose function is required for induced mutagenesis, is predicted to encode a nonessential DNA polymerase. J Bacteriol *171*, 5659-5667.

Morrison, A., Miller, E.J., and Prakash, L. (1988). Domain structure and functional analysis of the carboxyl-terminal polyacidic sequence of the RAD6 protein of Saccharomyces cerevisiae. Mol Cell Biol *8*, 1179-1185.

Mortensen, U.H., Bendixen, C., Sunjevaric, I., and Rothstein, R. (1996). DNA strand annealing is promoted by the yeast Rad52 protein. Proc Natl Acad Sci U S A *93*, 10729-10734.

Motegi, A., Liaw, H.J., Lee, K.Y., Roest, H.P., Maas, A., Wu, X., Moinova, H., Markowitz, S.D., Ding, H., Hoeijmakers, J.H., *et al.* (2008). Polyubiquitination of proliferating cell nuclear antigen by HLTF and SHPRH prevents genomic instability from stalled replication forks. Proceedings of the National Academy of Sciences of the United States of America *105*, 12411-12416.

Motegi, A., Sood, R., Moinova, H., Markowitz, S.D., Liu, P.P., and Myung, K. (2006). Human SHPRH suppresses genomic instability through proliferating cell nuclear antigen polyubiquitination. J Cell Biol *175*, 703-708.

Murakumo, Y., Ogura, Y., Ishii, H., Numata, S., Ichihara, M., Croce, C.M., Fishel, R., and Takahashi, M. (2001). Interactions in the error-prone postreplication repair proteins hREV1, hREV3, and hREV7. J Biol Chem *276*, 35644-35651.

Nakagawa, M., Takahashi, S., Tanaka, A., Narumi, I., and Sakamoto, A.N. (2011). Role of AtPolzeta, AtRev1, and AtPoleta in UV light-induced mutagenesis in Arabidopsis. Plant Physiol *155*, 414-420.

Nelson, J.R., Lawrence, C.W., and Hinkle, D.C. (1996a). Deoxycytidyl transferase activity of

yeast REV1 protein. Nature *382*, 729-731.

Nelson, J.R., Lawrence, C.W., and Hinkle, D.C. (1996b). Thymine-thymine dimer bypass by yeast DNA polymerase zeta. Science *272*, 1646-1649.

Ohmori, H., Friedberg, E.C., Fuchs, R.P., Goodman, M.F., Hanaoka, F., Hinkle, D., Kunkel, T.A., Lawrence, C.W., Livneh, Z., Nohmi, T., *et al.* (2001). The Y-family of DNA polymerases. Mol Cell *8*, 7-8.

Okada, T., Sonoda, E., Yoshimura, M., Kawano, Y., Saya, H., Kohzaki, M., and Takeda, S. (2005). Multiple roles of vertebrate REV genes in DNA repair and recombination. Mol Cell Biol *25*, 6103-6111.

Olsson, M., and Lindahl, T. (1980). Repair of alkylated DNA in Escherichia coli. Methyl group transfer from O6-methylguanine to a protein cysteine residue. J Biol Chem *255*, 10569-10571.

Otsuka, C., Kunitomi, N., Iwai, S., Loakes, D., and Negishi, K. (2005). Roles of the polymerase and BRCT domains of Rev1 protein in translesion DNA synthesis in yeast in vivo. Mutat Res *578*, 79-87.

Pages, V., Bresson, A., Acharya, N., Prakash, S., Fuchs, R.P., and Prakash, L. (2008). Requirement of Rad5 for DNA polymerase zeta-dependent translesion synthesis in Saccharomyces cerevisiae. Genetics *180*, 73-82.

Parker, J.L., and Ulrich, H.D. (2009). Mechanistic analysis of PCNA poly-ubiquitylation by the ubiquitin protein ligases Rad18 and Rad5. EMBO J *28*, 3657-3666.

Pastushok, L., Moraes, T.F., Ellison, M.J., and Xiao, W. (2005). A single Mms2 "key" residue insertion into a Ubc13 pocket determines the interface specificity of a human Lys63 ubiquitin conjugation complex. J Biol Chem *280*, 17891-17900.

Pastushok, L., and Xiao, W. (2004). DNA postreplication repair modulated by ubiquitination and sumoylation. Adv Protein Chem *69*, 279-306.

Paulovich, A.G., and Hartwell, L.H. (1995). A checkpoint regulates the rate of progression through S phase in S. cerevisiae in response to DNA damage. Cell *82*, 841-847.

Pegg, A.E. (1984). Methylation of the O6 position of guanine in DNA is the most likely initiating event in carcinogenesis by methylating agents. Cancer Invest *2*, 223-231.

Pickart, C.M. (2001). Ubiquitin enters the new millennium. Mol Cell *8*, 499-504.

Pickart, C.M., and Fushman, D. (2004). Polyubiquitin chains: polymeric protein signals. Curr Opin Chem Biol *8*, 610-616.

Pinto, A.L., and Lippard, S.J. (1985). Binding of the antitumor drug cis-diamminedichloroplatinum(II) (cisplatin) to DNA. Biochim Biophys Acta *780*, 167-180.

Reynolds, P., Koken, M.H., Hoeijmakers, J.H., Prakash, S., and Prakash, L. (1990). The rhp6+ gene of Schizosaccharomyces pombe: a structural and functional homolog of the RAD6 gene from the distantly related yeast Saccharomyces cerevisiae. EMBO J *9*, 1423-1430.

Ries, G., Heller, W., Puchta, H., Sandermann, H., Seidlitz, H.K., and Hohn, B. (2000). Elevated UV-B radiation reduces genome stability in plants. Nature *406*, 98-101.

Robzyk, K., Recht, J., and Osley, M.A. (2000). Rad6-dependent ubiquitination of histone H2B in yeast. Science *287*, 501-504.

Roest, H.P., Baarends, W.M., de Wit, J., van Klaveren, J.W., Wassenaar, E., Hoogerbrugge, J.W., van Cappellen, W.A., Hoeijmakers, J.H., and Grootegoed, J.A. (2004). The ubiquitin-conjugating DNA repair enzyme HR6A is a maternal factor essential for early embryonic development in mice. Mol Cell Biol *24*, 5485-5495.

Roest, H.P., van Klaveren, J., de Wit, J., van Gurp, C.G., Koken, M.H., Vermey, M., van Roijen, J.H., Hoogerbrugge, J.W., Vreeburg, J.T., Baarends, W.M., *et al.* (1996). Inactivation of the HR6B ubiquitin-conjugating DNA repair enzyme in mice causes male sterility associated with chromatin modification. Cell *86*, 799-810.

Rosentein, B.S., and J. M. Ducore (1983). Induction of DNA strand breaks in normal fibroblasts exposed to monochromatic ultraviolet and visible wavelenths in the 240-546 nm range. Photochem Photobiol *38*, 51-55.

Sakamoto, A., Lan, V.T., Hase, Y., Shikazono, N., Matsunaga, T., and Tanaka, A. (2003). Disruption of the AtREV3 gene causes hypersensitivity to ultraviolet B light and gamma-rays in Arabidopsis: implication of the presence of a translesion synthesis mechanism in plants. Plant Cell *15*, 2042-2057.

Sakumi, K., Shiraishi, A., Hayakawa, H., and Sekiguchi, M. (1991). Cloning and expresion of cDNA for rat O6-methylguanine-DNA methyltransferase. Nucleic Acids Res *19*, 5597-5601.

Sancar, G.B., Smith, F.W., and Heelis, P.F. (1987). Purification of the yeast PHR1 photolyase from an Escherichia coli overproducing strain and characterization of the intrinsic chromophores of the enzyme. J Biol Chem *262*, 15457-15465.

Santiago, M.J., Alejandre-Duran, E., and Ruiz-Rubio, M. (2006). Analysis of UV-induced mutation spectra in Escherichia coli by DNA polymerase eta from Arabidopsis thaliana. Mutat Res *601*, 51-60.

Sarkar, S., Davies, A.A., Ulrich, H.D., and McHugh, P.J. (2006). DNA interstrand crosslink repair during G1 involves nucleotide excision repair and DNA polymerase zeta. EMBO J *25*, 1285-1294.

Sedgwick, B., and Vaughan, P. (1991). Widespread adaptive response against environmental methylating agents in microorganisms. Mutat Res *250*, 211-221.

Shaked, H., Avivi-Ragolsky, N., and Levy, A.A. (2006). Involvement of the Arabidopsis

SWI2/SNF2 chromatin remodeling gene family in DNA damage response and recombination. Genetics *173*, 985-994.

Sharma, S., Hicks, J.K., Chute, C.L., Brennan, J.R., Ahn, J.Y., Glover, T.W., and Canman, C.E. (2011). REV1 and polymerase {zeta} facilitate homologous recombination repair. Nucleic Acids Res.

Singer, B., and J. T. Kusmierek (1982). Chemical mutagensis. Annu Rev Biochemistry *51*, 655-693.

Singhal, R.K., Hinkle, D.C., and Lawrence, C.W. (1992). The REV3 gene of Saccharomyces cerevisiae is transcriptionally regulated more like a repair gene than one encoding a DNA polymerase. Mol Gen Genet *236*, 17-24.

Spence, J., Sadis, S., Haas, A.L., and Finley, D. (1995). A ubiquitin mutant with specific defects in DNA repair and multiubiquitination. Mol Cell Biol *15*, 1265-1273.

Stelter, P., and Ulrich, H.D. (2003). Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation. Nature *425*, 188-191.

Sung, P., Prakash, S., and Prakash, L. (1988). The RAD6 protein of Saccharomyces cerevisiae polyubiquitinates histones, and its acidic domain mediates this activity. Genes Dev *2*, 1476-1485.

Takahashi, S., Sakamoto, A., Sato, S., Kato, T., Tabata, S., and Tanaka, A. (2005). Roles of Arabidopsis AtREV1 and AtREV7 in translesion synthesis. Plant Physiol *138*, 870-881.

Tateishi, S., Sakuraba, Y., Masuyama, S., Inoue, H., and Yamaizumi, M. (2000). Dysfunction of human Rad18 results in defective postreplication repair and hypersensitivity to multiple mutagens. Proc Natl Acad Sci U S A *97*, 7927-7932.

Thresher, R.J., Vitaterna, M.H., Miyamoto, Y., Kazantsev, A., Hsu, D.S., Petit, C., Selby, C.P., Dawut, L., Smithies, O., Takahashi, J.S., *et al.* (1998). Role of mouse cryptochrome blue-light photoreceptor in circadian photoresponses. Science *282*, 1490-1494.

Todo, T. (1999). Functional diversity of the DNA photolyase/blue light receptor family. Mutat Res *434*, 89-97.

Torres-Ramos, C.A., Yoder, B.L., Burgers, P.M., Prakash, S., and Prakash, L. (1996). Requirement of proliferating cell nuclear antigen in RAD6-dependent postreplicational DNA repair. Proc Natl Acad Sci U S A *93*, 9676-9681.

Tsui, C., Raguraj, A., and Pickart, C.M. (2005). Ubiquitin binding site of the ubiquitin E2 variant (UEV) protein Mms2 is required for DNA damage tolerance in the yeast RAD6 pathway. J Biol Chem *280*, 19829-19835.

Ulrich, H.D. (2003). Protein-protein interactions within an E2-RING finger complex. Implications for ubiquitin-dependent DNA damage repair. J Biol Chem *278*, 7051-7058.

Ulrich, H.D., and Jentsch, S. (2000). Two RING finger proteins mediate cooperation between ubiquitin-conjugating enzymes in DNA repair. EMBO J *19*, 3388-3397.

Unk, I., Hajdu, I., Fatyol, K., Hurwitz, J., Yoon, J.H., Prakash, L., Prakash, S., and Haracska, L. (2008). Human HLTF functions as a ubiquitin ligase for proliferating cell nuclear antigen polyubiquitination. Proc Natl Acad Sci U S A *105*, 3768-3773.

Unk, I., Hajdu, I., Fatyol, K., Szakal, B., Blastyak, A., Bermudez, V., Hurwitz, J., Prakash, L., Prakash, S., and Haracska, L. (2006). Human SHPRH is a ubiquitin ligase for Mms2-Ubc13-dependent polyubiquitylation of proliferating cell nuclear antigen. Proc Natl Acad Sci U S A *103*, 18107-18112.

Vairapandi, M., and Duker, N.J. (1994). Excision of ultraviolet-induced photoproducts of 5-methylcytosine from DNA. Mutat Res *315*, 85-94.

Vande Berg, B.J., and G. B. Sancar (1998). Evidence for dinucleotide flipping by DNA photolyase. J Biol Chem *273*, 20276-20284.

Wagner, J., Gruz, P., Kim, S.R., Yamada, M., Matsui, K., Fuchs, R.P., and Nohmi, T. (1999). The dinB gene encodes a novel E. coli DNA polymerase, DNA pol IV, involved in mutagenesis. Mol Cell *4*, 281-286.

Wang, H., Zhou, Y., Gilmer, S., Whitwill, S., and Fowke, L.C. (2000). Expression of the plant cyclin-dependent kinase inhibitor ICK1 affects cell division, plant growth and morphology. Plant J *24*, 613-623.

Watanabe, K., Tateishi, S., Kawasuji, M., Tsurimoto, T., Inoue, H., and Yamaizumi, M. (2004). Rad18 guides poleta to replication stalling sites through physical interaction and PCNA monoubiquitination. EMBO J *23*, 3886-3896.

Watkins, J.F., Sung, P., Prakash, S., and Prakash, L. (1993). The extremely conserved amino terminus of RAD6 ubiquitin-conjugating enzyme is essential for amino-end rule-dependent protein degradation. Genes Dev *7*, 250-261.

Wen, R., Newton, L., Li, G., Wang, H., and Xiao, W. (2006). Arabidopsis thaliana UBC13: implication of error-free DNA damage tolerance and Lys63-linked polyubiquitylation in plants. Plant Mol Biol *61*, 241-253.

Wen, R., Torres-Acosta, J.A., Pastushok, L., Lai, X., Pelzer, L., Wang, H., and Xiao, W. (2008). Arabidopsis UEV1D promotes Lysine-63-linked polyubiquitination and is involved in DNA damage response. Plant Cell *20*, 213-227.

Wittschieben, J.P., Reshmi, S.C., Gollin, S.M., and Wood, R.D. (2006). Loss of DNA polymerase zeta causes chromosomal instability in mammalian cells. Cancer Res *66*, 134-142.

Wu, X., Yamamoto, M., Akira, S., and Sun, S.C. (2009). Regulation of hematopoiesis by the K63-specific ubiquitin-conjugating enzyme Ubc13. Proc Natl Acad Sci U S A *106*, 20836-20841.

Xiao, W., Chow, B.L., Broomfield, S., and Hanna, M. (2000). The Saccharomyces cerevisiae RAD6 group is composed of an error-prone and two error-free postreplication repair pathways. Genetics *155*, 1633-1641.

Xiao, W., Chow, B.L., Fontanie, T., Ma, L., Bacchetti, S., Hryciw, T., and Broomfield, S. (1999). Genetic interactions between error-prone and error-free postreplication repair pathways in Saccharomyces cerevisiae. Mutat Res *435*, 1-11.

Xiao, W., Derfler, B., Chen, J., and Samson, L. (1991). Primary sequence and biological functions of a Saccharomyces cerevisiae O6-methylguanine/O4-methylthymine DNA repair methyltransferase gene. EMBO J *10*, 2179-2186.

Xiao, W., Lechler, T., Chow, B.L., Fontanie, T., Agustus, M., Carter, K.C., and Wei, Y.F. (1998). Identification, chromosomal mapping and tissue-specific expression of hREV3 encoding a putative human DNA polymerase zeta. Carcinogenesis *19*, 945-949.

Xin, H., Lin, W., Sumanasekera, W., Zhang, Y., Wu, X., and Wang, Z. (2000). The human RAD18 gene product interacts with HHR6A and HHR6B. Nucleic Acids Res *28*, 2847-2854.

Yamagata, Y., Kato, M., Odawara, K., Tokuno, Y., Nakashima, Y., Matsushima, N., Yasumura, K., Tomita, K., Ihara, K., Fujii, Y., *et al.* (1996). Three-dimensional structure of a DNA repair enzyme, 3-methyladenine DNA glycosylase II, from Escherichia coli. Cell *86*, 311-319.

Yamashita, Y.M., Okada, T., Matsusaka, T., Sonoda, E., Zhao, G.Y., Araki, K., Tateishi, S., Yamaizumi, M., and Takeda, S. (2002). RAD18 and RAD54 cooperatively contribute to maintenance of genomic stability in vertebrate cells. EMBO J *21*, 5558-5566.

Zhang, W., Qin, Z., Zhang, X., and Xiao, W. (2011). Roles of sequential ubiquitination of PCNA in DNA-damage tolerance. FEBS Lett.

Zhang, Y., Wu, X., Rechkoblit, O., Geacintov, N.E., Taylor, J.S., and Wang, Z. (2002). Response of human REV1 to different DNA damage: preferential dCMP insertion opposite the lesion. Nucleic Acids Res *30*, 1630-1638.

Zhao, X., Liu, J., Hsu, D.S., Zhao, S., Taylor, J.S., and Sancar, A. (1997). Reaction mechanism of (6-4) photolyase. J Biol Chem *272*, 32580-32590.

Zhou, Y., Wang, H., Gilmer, S., Whitwill, S., Keller, W., and Fowke, L.C. (2002). Control of petal and pollen development by the plant cyclin-dependent kinase inhibitor ICK1 in transgenic Brassica plants. Planta *215*, 248-257.

Zwirn, P., Stary, S., Luschnig, C., and Bachmair, A. (1997). Arabidopsis thaliana RAD6 homolog AtUBC2 complements UV sensitivity, but not N-end rule degradation deficiency, of Saccharomyces cerevisiae rad6 mutants. Curr Genet *32*, 309-314.